The Role of Fission Yeast F-box Protein Pof1 in Cell Growth Control

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Abstract

The ubiquitin-proteasome pathway is a central mechanism for regulating protein stability in eukaryotic cells. It involves the attachment of ubiquitin to a target protein marking it for degradation by the 26S proteasome. E3 ubiquitin ligases are an essential part of this process and one of the largest and most variable E3 ligase families is that of the SCF complexes. In order to study the role of the SCF complex in fission yeast, the role of Pof1, an essential fission yeast F-box protein, has been characterised. Temperature-sensitive *pof1* mutants were generated. At the restrictive temperature these display acute growth arrest with small cell size. Extragenic suppressor analysis identified Zip1, a bZIP transcription factor, as a target for Pof1. Zip1 is shown to be stabilised in *pof1* mutants, Pof1 binds only phosphorylated forms of Zip1, and Zip1 is ubiquitylated *in vivo*, indicating that Zip1 is a substrate of SCF^{Pof1}. Genome-wide DNA microarray assay shows that many cadmium-induced genes are under the control of Zip1, suggesting Zip1 plays a role in the cadmium response. Consistently, *zip1* mutants are hypersensitive to cadmium and unlike wild type, lose cell viability under this stress. Intriguingly, cadmium exposure results in upregulation of Zip1 levels and leads wild type cells to arrest growth with small cell size, reminiscent of *pof1* phenotypes. Our results indicate that Zip1 mediates growth arrest in response to cadmium, which is essential to maintain viability. Normally growing cells prevent this response through constitutive ubiquitylation and degradation of Zip1 via SCF^{Pof1}.

Dedication

This thesis is dedicated to my family.

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Abbreviations

APC	anaphase promoting complex
BSA	bovine serum albumin
Cdc	cell division cycle
Cdk	cyclin dependent kinase
CDK1	cyclin-dependent kinase 1
DAPI	diamidineophenylindole
DNA	deoxyribonucleic acid
dNTP	nucleotide triphosphates
E6-AP	E6-associated protein
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMM	Edinburgh minimal media
FACS	fluorescence-activated cell sorting
g	gram
G1/G2	gap1 or gap2
GFP	green fluorescent protein
GSH	glutathione
h	hours
HA	haemagglutinin A
HECT	homologous to E6-AP carboxy terminus
HIF	hypoxia inducibe factor
HPV	human papillomavirus
HU	hydroxyurea
IP	immunoprecipitation
mg	milligram
min	minute
ml	millilitre
mM	millimolar

M	molar
MMS	methyl methanesulphonate
MPF	maturation promoting factor
M-phase	mitotic phase
ng	nanogram
ORF	open reading frame
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
RING	really interesting new gene
RNA	ribonucleic acid
RPTK	receptor protein tyrosine kinases
S-phase	synthesis phase
SCF	Skp1-Cdc53-F-box
SDS	sodium dodecyl sulphate
SOCS	suppressor of cytokine signalling
tBOOH	tert-butylhydroperoxide
ts	temperature sensitive
UV	ultra violet
VBC	VHL-Elongin B- Elongin C Complex
VHL	Von Hippel-Lindau
YE5s	yeast extract 5 supplements
wt	wild type
Δ	deletion
μg	microgram

Chapter 1

Introduction

1.1 The importance of cell growth

The cell cycle is now understood to be a series of coordinated events, with initiation of one step often being dependent upon successful completion of some earlier step. Many of the events occurring in the cell cycle were elucidated in studies using yeast, where mutations in key regulators led to cells with uncoordinated cell cycles. It became clear from these studies that cells often used the irreversible destruction of some regulators as a mechanism to ensure cell cycle progression, thus proteolysis was recognised to be an important mechanism in cell cycle control.

However, although many of the regulators which control cell cycle progression are now understood, less is known about actual cell growth. Many early genetic studies in yeast showed that size controls exist within the cell which will alter the rate of cell cycle progression in response to changes in cell mass increase to maintain a constant cell size. However, the fundamental mechanisms controlling the mass increase itself are still not well understood. These mechanisms may be of even greater importance in higher organisms where there is some evidence that growth can be regulated independently of the cell cycle.

The work presented in this thesis will discuss a new role for proteolysis as not only a regulatory mechanism for cell cycle regulators but also for cell growth regulators. It will begin with a discussion of ubiquitin-mediated proteolysis, focusing on the role of the SCF ubiquitin ligase. The concept of cell growth

will then be examined and finally there will be an introduction to cell growth control, focussing on conditions when growth should be inhibited, especially during times of cell stress.

1.2 The Ubiquitin-proteasome pathway

The destruction of a protein is biologically no easy task. Peptide bonds are extremely stable under physiological conditions, an obviously desirable trait since it is difficult to imagine a cell functioning with proteins subject to random degradation. However, there are times when it is necessary for proteins to be destroyed. As well as needing to remove damaged proteins and recycle unnecessary proteins, proteolysis is also widely used by the cell as a control mechanism. This is seen most clearly in the cell cycle, where the destruction of key regulators ensures that they cannot act at the wrong time and gives directionality. In order to carry out this protein destruction the cell has developed proteases, which catalyse the hydrolysis of the peptide bond. However, these proteases must be highly selective, especially when it comes to the destruction of proteins such as cell cycle regulators, in which case not only the correct protein must be destroyed but at the correct time. In order to regulate proteolysis the cell has developed a system of marking proteins with a destruction tag, a highly conserved 76 amino acid polypeptide, ubiquitin. This ubiquitin tag then serves as a signal for the protein to be destroyed by the proteasome, a 2.5MDa proteolytic complex. This ubiquitin-proteasome pathway is one of the major pathways for intracellular proteolysis.

Ubiquitylation involves the covalent attachment of ubiquitin to a lysine side chain on the substrate through an isopeptide bond (Ciechanover, A. et al., 1980; Hershko, A. et al., 1980). This occurs through a well-characterised series of reactions (Figure 1.1). The ubiquitin is first activated on its C-

terminal glycine residue to a thiol ester intermediate. This is carried out by an ubiquitin-activating enzyme (E1) which uses ATP to synthesise ubiquitin adenylate, which then serves as a substrate for the formation of the ubiquitin thiol ester (Haas, A.L. and Rose, I.A., 1982). This ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2) and finally to the substrate via an ubiquitin ligase (E3) (Hershko, A. et al., 1983). The E3 enzyme may itself form an ubiquitin thiol ester in order to transfer the ubiquitin to the substrate or it may indirectly ubiquitylate the substrate by forming a platform for the E2 and substrate to interact, depending on its type (reviewed in Jackson, P.K. et al., 2000). The first ubiquitin moiety is transferred to the e-NH_2 group of the substrate. In successive reactions ubiquitin is attached to Lys48 of the previously conjugated ubiquitin molecule through its C-terminal Gly76 (Chau, V. et al., 1989), thus generating a polyubiquitin chain. Although in many cases the same E2/E3 is responsible for the initial ubiquitin ligation and these successive rounds of ubiquitylation, there have been some cases where these reactions have been catalysed by distinct complexes (Koegl, M. et al., 1999; Mastrandrea, L.D. et al., 1999). The presence of a polyubiquitin chain targets the substrate protein to the proteasome, which uses energy from ATP hydrolysis to unfold the substrate and feed it into its proteolytically active core (Glickman, M.H. et al., 1998). Here the substrate is hydrolysed into small peptides and ubiquitin, having been released from the substrate by deubiquitylation enzymes, can be recycled (Figure1.2). How the ubiquitylated substrate is targeted to the proteasome is still not clear, although recent data shows E3 ligases themselves can interact with the proteasome, suggesting a model where, after tagging a protein for degradation, the E3s may go even further and deliver it to the degradation site (Farras, R. et al., 2001; Verma, R. et al., 2000; Xie, Y. and Varshavsky, A., 2000).

Ubiquitylation without destruction

It is important to note that the ubiquitin modification of a protein does not always serve as a degradation signal. Many examples have been found of proteins whose ubiquitylation is carried out by an E3 ubiquitin ligase but this leads to some modification of the protein function rather than its destruction. In particular, many transcriptional activators appear to fall into this class of substrate. It was noticed that many transcriptional activators contain overlapping transcriptional activation domains and degrons, the regions required for ubiquitylation (Molinari, E. et al., 1999). It was later shown that the reason for this in some cases appeared to be because ubiquitylation acted as an activation signal (Kim, S.Y. et al., 2003; Salghetti, S.E. et al., 2001). In the case of the oncoprotein transcription factor Myc it has been suggested that ubiquitylation by SCF^{Skp2} is required for its activation and then later its destruction (Kim, Herbst et al. 2003). How a protein can carry an ubiquitin modification but not get degraded by the proteasome remains a mystery. In some cases these proteins are only modified by a single ubiquitin residue; monoubiquitylation acts as a signal for protein sorting, for example (Hicke, L. and Dunn, R., 2003) and presumably a single ubiquitin residue cannot be recognised by the proteasome. In other cases an ubiquitin chain is linked through ubiquitin Lys63 rather than Lys48. The DNA polymerase processivity factor PCNA can be modified in this manner and this causes it to carry out error-free, replicative DNA legion bypass in response to DNA damage (Hoege, C. et al., 2002). It was thought that these non-canonical ubiquitin modifications might be non-degradative, whilst canonical Lys48-linked ubiquitin chains may lead to proteasome recognition and degradation. However it was recently shown that Met4, a budding yeast transcription factor involved in methionine biosynthesis (see below) could be modified by a Lys48- linked ubiquitin chain and this acted as a non-degradative signal to inhibit Met4 activity (Flick, Ouni et al. 2004). Thus it is still unclear how some ubiquitin chains can alter a protein's activity without leading to its degradation.

The variability of the ubiquitin ligases

One of the most amazing facts to emerge from studies of the ubiquitin pathway is the sheer breadth of the system. There are approximately fifty E2 enzymes in humans and the number of E3s is even greater, with even conservative estimates in humans being in the hundreds. The organisation of the system is hierarchical. In the yeast *Saccharomyces cerevisiae,* as in humans, a single E1 enzyme activates ubiquitin (McGrath, J.P. et al., 1991) for thirteen E2 enzymes which in turn ubiquitylate substrates via an even greater number of ubiquitin ligases. This manner of organisation means that not only can many different substrates be ubiquitylated by the same pathway but allows regulatory potential which is economical to the cell: the ubiquitylation of a specific substrate can be switched on or off at the level of the ligase, without affecting the overall ability of the cell to carry out ubiquitylation. This system depends upon there being a large number of E3s, with each one recognising a restricted set of substrates.

1.3 The E3 Ubiquitin ligases

All E3 ligases characterised so far contain one of two domains; an approximately 350 amino acid HECT (homologous to E6-AP carboxy terminus) domain or a RING (really interesting new gene) finger domain (reviewed in Pickart, C.M., 2001) (Figure 1.3). Recent reports suggest there may be a third family of U-box E3s, containing a RING finger-related domain (Hatakeyama, S. et al., 2001; Koegl, M. et al., 1999). However, the biological function of these U-box proteins as E3s remains to be characterised.

The HECT domain E3 ubiquitin ligases

The discovery of the HECT domain came about from studies of the selective degradation of the p53 tumour suppressor. The infection of cells with oncogenic forms of human papillomavirus (HPV) leads to the selective degradation of p53 via ubiquitin-mediated proteolysis (Scheffner, M. et al., 1990). This ubiquitylation was induced by the virus itself and depended upon the HPV E6 gene product and a cellular protein called E6-AP (E6-associated protein). E6 recruits E6-AP to form a p53 specific ubiquitin ligase (Scheffner, M. et al., 1993). The C-terminal region of E6-AP was found to contain a 350 amino acid region with high sequence similarity to a number of other proteins, appropriately named the HECT domain (Huibregtse, J.M. et al., 1995). A conserved cysteine residue within the HECT domain is essential for binding to ubiquitin. This residue forms an ubiquitin thiol ester (Scheffner, M. et al., 1995) from which ubiquitin is directly transferred to the substrate.

Substrate binding and recognition

HECT domain ligases all appear to be of similar modular construction, consisting of a unique substrate binding N-terminal domain linked to the well-conserved, catalytic HECT domain at the C-terminus (Figure 1.3A). Although a large number of potential HECT E3s exist in databases very few have been characterised. Those that have appear to bind to a varied list of substrates (Table 1). Ubiquitin ligases often contain recognised protein-protein interaction motifs in their substrate binding domains. So far only WW domains, phosphoserine or phosphothreonine binding motifs (Lu, P.J. et al., 1999) which can also bind to PPXY motifs in target proteins (Nguyen, J.T. et al., 1998), have been identified in HECT E3s. These were first found in the N-terminal substrate binding domain of a well characterised HECT E3, *S. cerevisiae* Rsp5 (Wang, G. et al., 1999). The WW domains in Rsp5 are essential for binding to its substrate, Rpb1, the large subunit of RNA polymerase II (Huibregtse, J.M. et al., 1997; Wang, G. et al., 1999). Rsp5

binds to and ubiquitylates Rpb1 upon DNA damage, leading to its degradation (Beaudenon, S.L. et al., 1999). Rsp5 illustrates another characteristic of HECT domain E3s; they are able to have more than one substrate. In addition to Rpb1, Rsp5 ubiquitylates a set of plasma membrane permeases and receptors, leading to their endocytosis (reviewed in (Rotin, D. et al., 2000). It is also responsible for the activation of two transcription factors, Spt23 and Mga2, by ubiquitylating precursor forms of these proteins, leading to their processing by proteasomes and the production of active forms (Hoppe, T. et al., 2000).

Studies of Rsp5 and its human homologue Nedd4 have revealed most of what is known so far about how HECT ligases recognise substrates. Rsp5, Nedd4 and a more recently characterised HECT E3, Smurf1, all seem to rely on SPTSPSY repeats or PPXY motifs for substrate recognition, via their WW domains (Huibregtse, Yang et al. 1997; Zhu, H. et al., 1999). Only one other mode of interaction has so far been identified and this is between E6-AP and one of its substrates Mcm7, which interact using a short motif named the L2G box that is conserved between them (Kuhne, C. and Banks, L., 1998). There has been one case of substrate phosphorylation being a prerequisite for ubiquitylation but so far this does not appear to be as common for HECT E3s as for RING finger ubiquitin ligases (see later discussion). Although there is direct interaction between the N-terminal domain of a HECT E3 and its substrate, recent studies have also suggested that other proteins may be involved in adapting the ligase Rsp5 to other substrates (Hettema, E.H. et al., 2004). How widely conserved all of these mechanisms are in HECT E3-substrate recognition remains to be determined.

The RING finger E3 ubiquitin ligases

Unlike the HECT domain E3s, RING finger ubiquitin ligases do not appear to form a direct thiol ester with ubiquitin. The RING finger domain consists of an

octet of cysteine and histidine residues arranged with characteristic spacing which allows for the coordination of two zinc ions forming a cross-brace structure. In some cases this RING finger protein contains a substrate binding domain and is able to function alone as a single-subunit E3 (See Table 2 for examples of such ubiquitin ligases). In another family of RING E3s the RING finger protein is just one of multiple subunits (reviewed in (Joazeiro, C.A. and Weissman, A.M., 2000). However all types of non-HECT E3s characterised so far appear to contain this RING finger domain suggesting that it is central to catalysis. Although the exact mechanism of RING E3 ubiquitylation is not yet known, experiments on a multisubunit E3, the SCF (see below for description of the SCF), have shown that the RING finger domain along with another subunit called a cullin, is able to bind to the E2 and promote ubiquitin polymerisation and that this activity is dependent upon the zinc-coordinating residues of the RING domain and is insensitive to thiol-modifying agents (Seol, J.H. et al., 1999). These data suggest that the RING domain is able to catalyse ubiquitylation without binding ubiquitin directly, possibly acting as a scaffold and bringing the E2 and substrate together directly (Borden, K.L., 2000; Zheng, N. et al., 2000).

More evidence for this model comes from the crystal structure of RING E3/E2 complexes. The structure of a fragment of the RING E3 c-Cbl with its E2 UbcH7 has been solved (Zheng, N. et al., 2000). c-Cbl ubiquitylates activated receptor protein tyrosine kinases (RPTKs) leading to their degradation and thus terminating signalling (Joazeiro, C.A. et al., 1999; Waterman, H. et al., 1999). Epidermal growth factor (EGF) receptor (EGFR) is one of these substrates. Deletions and mutations in the c-Cbl RING domain abolish EGF dependent EGFR degradation, with some of these mutant c-Cbl proteins becoming oncogenic (Waterman, H. et al., 1999). This is further evidence that the RING domain is necessary for ubiquitin-ligase activity. The crystal structure of c-Cbl with its E2 enzyme UbcH7 shows that UbcH7 interacts with

the RING domain and a small region of flanking residues. The active site of the E2 is 15Å away from the closest RING side-chain, suggesting that the thiol ester does not directly transfer to the RING domain. From the structural studies it appears most likely that it is the induced proximity between the substrate and the E2 which leads to ubiquitylation.

The multisubunit ubiquitin ligases

Multisubunit E3s take the modular design of HECT and single subunit RING E3s one step further, using separate protein components for their substrate-binding and catalytic functions. Three types of multisubunit E3s are well characterised: SCF (Skp1- Cullin- F-box protein) complexes, the APC (Anaphase Promoting Complex) and the VBC (VHL- elongin B- elongin C) complex. All three types are built around a similar modular design, with each containing a small RING finger protein and a substrate recruiting protein built around a cullin-family protein which acts as a molecular scaffold (Figure 1.3). Two further cullin-based ubiquitin ligases have been recently identified; BTB-Cullin3-Roc1 ligases and Cullin4-Roc1 ligases (Furukawa, M. et al., 2003; Higa, L.A. et al., 2003). So far only a limited number of reactions in which these complexes are involved have been identified but the large number of BTB-domain-containing proteins found in the genome suggests that BTB-Cullin3-Roc1 ligases at least may represent a new multisubunit E3 family (Krek, W., 2003; Pintard, L. et al., 2004).

The Anaphase Promoting Complex

The APC was the first multisubunit ubiquitin ligase to be discovered. It controls the metaphase to anaphase transition during mitosis and helps establish and maintain the G1-phase of the cell cycle (reviewed in (Harper, J.W. et al., 2002; Zachariae, W. and Nasmyth, K., 1999). The main APC targets during mitosis are securin, whose destruction promotes sister chromatid separation and B-type cyclins, whose destruction is essential for

inactivation of mitotic CDKs and exit from mitosis. The APC is a structurally complex E3, having at least 11 subunits in vertebrates and 13 in yeast (see above reviews and (Hall, M.C. et al., 2003; Irniger, S. et al., 1995; Passmore, L.A. et al., 2003; Yoon, H.J. et al., 2002; Zachariae, W. et al., 1998b) (Figure 1.3E). The RING finger subunit, APC11, interacts with a cullin domain in APC2, forming the RING finger-cullin backbone found in all multiprotein E3s. This complex alone is able to bind E2 enzymes (Tang, Z. et al., 2001). The role of the other APC subunits is less understood. Much evidence is now arising to suggest that Apc10/Doc1 plays a role in substrate recognition (Passmore, L.A. et al., 2003). This role was originally believed to be carried out by additional APC activator proteins, Cdc20 and Cdh1 (Fang, G. et al., 1998; Kramer, E.R. et al., 2000; Schwab, M. et al., 1997; Visintin, R. et al., 1997). These proteins bind to the APC in a cell cycle regulated manner. Cdc20 associates with the APC following mitotic phosphorylation of APC subunits. This APC^{Cdc20} complex is able to degrade mitotic substrates including B-type cyclins (Dawson, I.A. et al., 1993; Sigrist, S. et al., 1995), leading to inactivation of cyclin dependent kinase 1 (CDK1). This inactivation leads to the removal of an inhibitory phosphorylation on Cdh1, which allows its association with the APC during late mitosis and G1/G0 (Jaspersen, S.L. et al., 1999; Schwab, M. et al., 1997; Sorensen, C.S. et al., 2000; Sorensen, C.S. et al., 2001; Zachariae, W. et al., 1998a). Cdc20 and Cdh1 have both been shown to be required for the degradation of specific APC substrates. In budding yeast Cdc20 is required for Pds1 (securin) but not Clb2 (cyclin) degradation, yet Cdh1 is required for Clb2 degradation, but not Pds1. Along with the observation that Cdc20 and Cdh1 can directly bind to APC substrates and are required for APC-substrate association (Burton, J.L. and Solomon, M.J., 2001; Hilioti, Z. et al., 2001; Pfleger, C.M. et al., 2001; Schwab, M. et al., 2001), this led to the idea that these coactivators act as substrate specific recruitment factors for the APC, similar to the role of F-box proteins within the SCF. Current data would suggest that Apc10/Doc1 also

contributes to substrate recognition, possibly helping Cdc20/Cdh1 recruit specific substrates to the APC. Aside from Apc10, roles of the other APC subunits appear to be more structural. A biochemically purified APC2/11, APC1, APC4, APC5 complex was shown to be able to form multiubiquitin chains but not recruit substrates and a similarly purified complex containing all subunits except APC2/11 could recruit substrates but not support *in vitro* ubiquitylation (Vodermaier, H.C. et al., 2003). Two subunits containing tetratrico peptide repeats (TPRs), APC3 and APC7 were able to bind Cdh1 and Cdc20 (Vodermaier, H.C. et al., 2003). This data suggests that the role of the TPR proteins (APC3, APC6, APC7 and APC8) within the APC is to recruit regulatory proteins such as Cdc20, and thus substrates, to the APC complex. APC1, APC4 and APC5 may then connect APC2/11 with these TPR subunits.

The VHL- Elongin B- Elongin C Complex

The VBC ubiquitin ligase is structurally similar to the SCF complex, having far less subunits than the APC (Figure 1.3C). Like the APC and SCF complexes it contains a RING finger protein, Roc1/Rbx1 and a cullin protein, Cul2. The VBC complex is so named because it was first identified in complex with the VHL (Von Hippel-Lindau) tumour suppressor protein. Von Hippel-Lindau disease is a dominant inherited syndrome which is characterised by the predisposition to develop various types of tumour. It was found to be caused by mutations in the VHL tumour suppressor gene. Cells containing mutations in this gene were shown to express high levels of hypoxia-induced mRNAs which lead to their hypervascularity and presumably neoplasmic tendencies. One of the reasons for this was that hypoxia-inducible factors HIF1 and HIF2, transcription factors for these hypoxia-induced genes, were found to be degraded in a VHL-dependent manner. VHL itself was then shown to act as an ubiquitin ligase with the Cul2/ Roc1 complex (Iwai, K. et al., 1999). Thus VHL is believed to be the substrate recruitment subunit of the VBC complex. In addition to the RING finger-cullin backbone and the substrate recruiting

VHL component, two further VBC subunits are known, Elongin B and Elongin C. Both were named after their discovery in a complex controlling transcriptional elongation. Elongin B is homologous to ubiquitin but its role in the VBC complex is unclear. Elongin C is homologous to the SCF Skp1 component and like Skp1 connects the substrate-adaptor protein, in this case VHL, to the core complex. VHL binds to Elongin C through a motif called the BC box. This motif is found in other proteins, including members of the suppressor of cytokine signalling (SOCS) family, where it is within the SOCS box domain. It is possible that this motif acts like an F-box (see below), allowing different adaptor proteins to interact and recruit substrates for the VBC complex through Elongin C interactions. However VHL is so far the only substrate recruitment subunit to be found acting with the Elongin C/ Cul2/ Roc1 complex.

The SCF complex

SCF ubiquitin ligases consist of at least 4 subunits: Skp1, Cul1 (or Cdc53), Roc1/Rbx1/Hrt1 and an F-box protein (Figure 1.3D). Like the APC and VBC complexes, the core of the SCF complex consists of the catalytic RING finger protein, Roc1 and the cullin scaffold, Cul1. SCF complexes recruit their substrates through F-box proteins; adaptor proteins containing a conserved, approximately 45 amino acid motif called the F-box, which mediates binding of the F-box protein and substrate to the core complex through Skp1, which in turn associates with the N-terminus of Cul1. It seems from *in vitro* biochemical studies that these four components alone are enough to reconstitute SCF ubiquitylation activity towards specific substrates (Feldman, R.M. et al., 1997; Skowyra, D. et al., 1997). However other components may be present in the complex, such as Sgt1 (Kitagawa, K. et al., 1999), which has been shown to interact with the SCF and be required for the turnover of some substrates. It is possible certain components necessary *in vivo* have been missed in these reconstitution assays.

The identification of the SCF ubiquitin ligase complex came from work in budding yeast analysing the G1/S-phase transition. Mutants which could not progress from G1 to S-phase; *cdc4*, *cdc34* and *cdc53*, were shown to be defective in this process due to an accumulation of an S-phase inhibitor Sic1 (Schwob, E. et al., 1994) and later this was also shown to be the case in the *skp1* mutant (Bai, C. et al., 1996). Cdc34 (also known as Ubc3) was already known to have ubiquitin-conjugating activity (Goebl, M.G. et al., 1988) so it was obvious how this could be acting to destabilise Sic1, but the role of the other proteins in this process was unclear. Cdc53 and Cdc4 were shown to precipitate with Cdc34 from yeast cell lysates (Mathias, N. et al., 1996), suggesting that they may have a direct role in ubiquitylation. Independently Cdc53 was shown to precipitate with the G1 cyclin Cln2 and be necessary for its turnover (Willems, A.R. et al., 1996). Finally *SKP1* was identified in a screen for genes which could suppress *cdc4* mutants if overexpressed (Bai, C. et al., 1996), along with the gene which encodes human cyclin F (Bai, C. et al., 1994). The Cdc4 and human cyclin F proteins were shown to share a sequence motif named the F-box and this was shown to mediate their binding to Skp1 (Bai, C. et al., 1996). The SCF identification story was completed when all of these components were put together *in vitro* and it was shown that Sic1 ubiquitylation could be reconstituted. Firstly it was shown that Sic1 ubiquitylation in crude yeast extracts depended upon Cdc34, Cdc4 and G1 cyclin/CDK activity (Verma, R. et al., 1997). Then Cdc4, Cdc53 and Skp1 expressed in insect cells were shown to assemble into a complex which when purified could promote ubiquitylation of phosphorylated Sic1 by Cdc34 conjugating enzyme (Feldman, R.M. et al., 1997; Skowyra, D. et al., 1997). The fourth SCF component, Hrt1/Roc1/Rbx1 was identified more recently in various immunoprecipitation experiments, as a protein which interacted with SCF^{Skp2} complex (Tan, P. et al., 1999), hCul1(Ohta, T. et al., 1999), Cdc53 (Seol, J.H. et al., 1999) and VBC complexes (Kamura, T. et al., 1999). It was shown in these studies that Hrt1 was required for Sic1 turnover, *hrt1*

temperature sensitive (ts) mutants accumulate Sic1 and *in vitro* Hrt1 stimulates the ability of the SCF to ubiquitylate Sic1. Thus Hrt1 was identified as another SCF component. The original SCF^{Cdc4} reconstitution assays were carried out without the addition of Hrt1 yet still worked, probably because the components were purified from insect cells, and Hrt1 is highly conserved, thus insect homologues were likely to have been incorporated into the SCF complexes. This illustrates that reconstitution assays are not always a reliable indicator of minimum complex components, thus as mentioned above there is a possibility that further SCF components will be identified in the future.

1.4 The F-box proteins

The discovery of Skp1 and the fact that it indirectly bound to SCF substrates via F-box motif-containing proteins lead to the proposal that F-box proteins act as adaptors between the ubiquitylation machinery and different substrates (Bai, C. et al., 1996). This was later confirmed when it was shown that three yeast F-box proteins, Cdc4, Grr1 and Met30 formed individual complexes with the Cdc34-Cdc53-Skp1 complex, and these F-box proteins were functionally specific *in vivo*, required for Sic1 degradation, Cln2 degradation and methionine gene repression respectively, whereas the core complex was required for all three functions (Patton, E.E. et al., 1998). This resulted in the 'F-box hypothesis' which suggested that the core SCF complex could be adapted to specific functions via F-box proteins, which contained an F-box motif to bind to Skp1 but a variable substrate binding domain (Patton, E.E. et al., 1998).This is an attractive model for SCF function, providing an economical method of ubiquitylating a large variety of substrates without the need to produce an equal number of different ubiquitylation complexes, since only the adaptor protein need be changed. Since this hypothesis was

suggested many F-box proteins have been shown to form functionally distinct SCF complexes in many different organisms (reviewed in Deshaies, R.J., 1999) and the number of proteins containing an F-box motif found in eukaryotic genomes suggests that a potentially vast number of SCF complexes and substrates may exist. However, it is important to note a couple of exceptions to the hypothesis which have arisen. Firstly, not all proteins containing an F-box motif may function within an SCF complex. Ctf13 is a budding yeast protein containing a highly divergent F-box motif but which binds to Skp1. However, Ctf13 is a component of the CBF3 kinetochore complex, which binds microtubules to the condensed mitotic chromosomes. Ctf13 requires interaction with Skp1 for assembly into this complex but no other SCF components appear to be present (Kaplan, K.B. et al., 1997; Russell, I.D. et al., 1999). Other examples of proteins containing an F-box which have been shown to function outside of an SCF complex include the *C. elegans* protein FOG-2 (Clifford, R. et al., 2000) and *X. laevis* and human cyclin F (Kong, M. et al., 2000). Although finding an F-box protein functioning outside of an SCF complex does not rule out the possibility it may have some other function which is within an SCF complex, it should be remembered that the phenotype caused by removal of an F-box protein from the cell may not be due to lack of a specific SCF function. Secondly, not all SCF complexes may contain only one F-box protein. Studies in *S. pombe* have revealed that some F-box proteins can form dimers. Pop1 and Pop2 are *S. pombe* F-box proteins homologous to *S. cerevisiae* Cdc4 (see below). It has been shown that they can form homo- and heterodimers and this interaction is essential for their function (Kominami, K. et al., 1998; Wolf, D.A. et al., 1999). It is not known if the dimers form within a monomeric or multimeric SCF complex.

1.5 Regulation of SCF ubiquitylation activity

F-box proteins generally seem to require phosphorylation of their substrate on a specific residue for recognition (Deshaies 1999). This creates an opportunity for multiple substrates of the same SCF complex to be regulated differently. SCF^{Cdc4}, for example, degrades substrates Sic1 and Far1 following their phosphorylation by G1 cyclin/Cdc28 (Feldman, R.M. et al., 1997; Skowyra, D. et al., 1997) but degrades Cdc6 following its phosphorylation by S-phase cyclin/Cdc28 (Elsasser, S. et al., 1999). Thus phosphorylation of substrates by different kinases allows temporal control of degradation. Whether all SCF complexes will require substrate phosphorylation remains to be seen. Those studied so far which have been shown to require substrate phosphorylation all contain either a WD-40 repeat or leucine –rich repeat substrate binding domain. It will be interesting to see if F-box proteins with structurally different binding domains also require substrate phosphorylation.

Aside from phosphorylation it is possible that availability of a specific F-box protein is another mechanism used to control the activity of an SCF complex. Several F-box proteins have been shown to be intrinsically short-lived proteins due to their own autoubiquitylation and this is essential for cell growth (Zhou, P. and Howley, P.M., 1998). It seems that this may be regulated by substrate availability, recent studies using the Homologue of Slimb (HOS) F-box protein showed that its *in vitro* ubiquitylation was dependent both upon its ability to interact with the core SCF components and inhibited by the presence of its phosphorylated substrate (Li, Y. et al., 2004). These data suggest a model where F-boxes bound to the SCF core complex will be degraded if there is no phosphorylated substrate for them to recruit. This mechanism would maintain the activity of specific SCF complexes based on the availability of a particular substrate. It has been shown in *S. pombe* that the overexpression of F-box protein Pof10 is lethal because it sequesters the core SCF components away from the essential F-box protein Pop1,

presumably resulting in the lethal accumulation of Pop1 substrates Rum1 and Cdc18 (Ikebe, C. et al., 2002). This suggests that the availability of some core SCF component is limited and that turnover of F-box proteins may also be necessary to release this core component from complexes which are not needed.

Thus from the small number of F-box proteins characterised so far it appears that regulation occurs mainly at the substrate-F-box protein interaction level. If a substrate is present and has been phosphorylated an F-box protein will bind to it and it will be degraded. No evidence of higher regulation such as switching on or off of the SCF complex itself has been found. This mode of regulation seems to differ from that of the other major multisubunit E3 ligase, the APC, where regulation of the whole complex seems to occur by phosphorylation and the association of the co-activator proteins Cdc20 and Cdh1 (see above). If phosphorylation occurs and co-activator proteins are bound to the APC it seems that the complex is active and all substrates will be degraded. It is possible that this difference in regulation is due to different functions of the two complexes. The APC is primarily a cell cycle regulator and as such must only be active during specific periods of the cell cycle. There is no reason for APC activity during S-phase for example, thus it can be switched off completely during these times. Studies of the SCF on the other hand have revealed that it is involved more diverse functions which could be needed at any point during the cell cycle. Roles such as the modulation of Met4 activity (see below) in response to environmental changes could be required at any time during the life of the cell, thus to switch off the SCF in a manner similar to the APC could be dangerous and as a result cells have evolved to regulate degradation at the level of the substrate, keeping the core SCF components constitutively active.

1.6 The SCF complex in *S. pombe*

All components of the SCF identified so far appear to be conserved within the fission yeast genome, including at least sixteen F-box motif containing proteins identified by genetic analysis and sequence homology (Hermand, D. et al., 2003; Ikebe, C. et al., 2002; Katayama, S. et al., 2002; Kominami, K. et al., 1998; Lehmann, A. et al., 2004) (Table 3). To date, only two of these F-box proteins have had their substrates elucidated, these are Pop1 and Pop2 (Kominami, K. and Toda, T., 1997). Pop1 and Pop2 are homologues of budding yeast Cdc4. They were originally identified when *pop1* mutants were isolated in a screen for polyploid mutants, and Pop1 was shown to be necessary for the degradation of the CDK inhibitor Rum1 and the S-phase regulator Cdc18. Hence *pop1* mutants accumulated Rum1, causing them to bypass mitosis, yet went through multiple S-phases due to Cdc18 accumulation. Later the closely related *pop2* was identified from sequence homology and it was shown that *pop2* mutants also accumulated Rum1 and Cdc18, and that Pop1 and Pop2 formed hetero- and homodimers (Jallepalli, P.V. et al., 1998) and had non-redundant roles in substrate degradation (Wolf, D.A. et al., 1999) suggesting that they worked cooperatively to recruit substrates. More recently it has been shown that the level of S-phase cyclin Cig2 is also controlled by $SCF^{Pop1/}$Pop2. Interestingly this occurs at the level of transcription as well as degradation (Yamano, H. et al., 2000; Yamano, H. et al., 2004).

As summarised in Table 1, of the other fourteen remaining *S. pombe* F-box proteins only two, Pof1 and Pof6, are completely essential to growth and only two others, Pof3 and Fdh1, appear to have any discernable phenotype when deleted (Katayama, Kitamura et al. 2002(Lehmann, A. et al., 2004). This suggests that either the other ten are only required to degrade some

substrate during exposure to specific conditions such as extracellular stress, or there is functional redundancy between these F-box proteins. Pof1 and Pof6 are both essential for cell growth. The role of neither has been elucidated, although mutant analysis and localisation studies suggest Pof6 has some role in cytokinesis (Hermand, D. et al., 2003).

Pof1 and its *S. cerevisiae* homologue Met30

Pof1 is homologous to the well-characterised budding yeast F-box protein Met30. *MET30* was originally identified in a screen for mutants defective in sulphur amino acid metabolism (Figure 1.4) (Thomas, D. et al., 1995); strains carrying a dominant negative *MET30* allele could not repress the methionine biosynthesis gene *MET25* in response to high levels of extracellular methionine. Later Met30 was shown to form a complex with the core SCF components and mutants in these components were also shown to be defective in *MET25* expression, suggesting that SCF^{Met30} activity was responsible for *MET* gene repression in response to methionine (Patton, E.E. et al., 1998). The mechanism by which SCF^{Met30} repressed the *MET* genes became clear when it was shown that the essential substrate of this complex was Met4, a transcriptional activator which regulates the *MET* gene network (Rouillon, A. et al., 2000). In the presence of methionine cells switch off Met4 activity through SCF^{Met30} (Kaiser, P. et al., 2000; Rouillon, A. et al., 2000) thus switching off methionine biosynthesis. The mechanism by which Met4 activity is switched off, however, remains controversial. There is evidence that in the presence of methionine Met4 is ubiquitylated and degraded in a Met30-dependent fashion, where Met30 can detect methionine availability through the levels of a downstream product of this amino acid, AdoMet, and adjust its activity accordingly (Patton, E.E. et al., 2000; Rouillon, A. et al., 2000). However, there is also data to suggest that, in the presence of methionine, Met4 is ubiquitylated, again through the actions of Met30, but this leads to

inhibition of Met4 activity without proteolysis (Kaiser, P. et al., 2000). Interestingly, this ubiquitylation appears to be a chain of ubiquitin moieties linked through their lysine 48 residues, the canonical proteasome recognition signal, leading to more questions about how this signal switches off Met4 without leading to its degradation (Flick, K. et al., 2004). The issue of whether Met4 regulation is proteolysis dependent or independent has been suggested to have arisen because Met4 regulation changes depending upon the media used to grow the cells being studied (Kuras, L. et al., 2002). However even this hypothesis is disputed, and to date Met4 regulation remains contentious. Another interesting aspect to the role of Met30 in the cell is that *MET30* mutants, as well as being unable to switch off the *MET* genes, show a G1-phase cell cycle arrest. This appears to be Met4 dependent but *MET* gene independent (Patton, E.E. et al., 2000).

1.7 Cell Growth and Cell-Cycle Progression

Cell growth, or cell mass increase, is as fundamental to organism growth as cell division. However, compared to our understanding of the cell division process, knowledge of the processes regulating cell growth is severely lacking. Many of the early studies of cell division using fission yeast were based on the isolation of mutant strains in which the coordination between cell growth and cell division was disrupted. The fission yeast cell cycle is similar to other eukaryotes, and can be divided into G1, S, G2 and M phase (Mitchison, J.M. and Creanor, J., 1971). S-phase, during which DNA is synthesised, is short and occurs at the beginning of the cell cycle simultaneously with septum formation. This is followed by a long G2-phase and then nuclear division, M-phase, which occurs at 0.75 of a cell cycle. Cell division is marked by septum formation across the middle of the cell. There is

a short G1 period and the cells will enter S-phase of the next cell cycle before the two equally sized daughter cells separate. During the cell cycle, cell diameter remains constant and cells grow only in length, thus cell length is directly related to cell cycle stage. This fact was exploited in early screens when mutants that had lost cell growth and cell cycle coordination were isolated by selecting cells that divided at different lengths to wild type (Nurse, P., 1975). Cloning of these mutant genes and elucidation of the functions of their products has led to a relatively good understanding of how fission yeast controls progression through the cell cycle, particularly mitosis. The Cdc2 kinase is the major element controlling the transitions through the cell cycle (Nurse, P. and Thuriaux, P., 1980; Nurse, P. et al., 1976; Thuriaux, P. et al., 1978). This acts in concert with cyclins for full kinase activity (Fisher, D. and Nurse, P., 1995). Cdc13 is a mitotic cyclin with which Cdc2 must function for mitotic progression (Booher, R. and Beach, D., 1987; Booher, R. and Beach, D., 1988; Hagan, I. et al., 1988). Together Cdc2 and Cdc13 constitute M-phase promoting factor (MPF). Fluctuations in MPF drive the cell cycle; it must be low during G1 and high at M-phase (reviewed Nurse, P., 1994). In terms of the regulation between cell growth and cell division the G2/ M phase transition represents a crucial control point, since once a cell has committed to division the size of its daughter cells cannot be increased any further. Thus there is tight regulation to ensure that MPF activity is only allowed to increase to maximum levels once a sufficient cell mass is reached. This acts through regulators of MPF, principally Wee1, Mik1 and Cdc25. Wee1 and Mik1 are tyrosine kinases which phosphorylate Tyr-15 of Cdc2, resulting in its inactivation (Featherstone, C. and Russell, P., 1991; Lee, M.S. et al., 1994; Lundgren, K. et al., 1991; Parker, L.L. and Piwnica-Worms, H., 1992). This action is antagonized by Cdc25 tyrosine phosphatase which dephosphorylates Tyr-15, activating Cdc2 (Millar, J.B. et al., 1991; Millar, J.B. and Russell, P., 1992; Russell, P. and Nurse, P., 1986).

Cell size controls

The cell size at which MPF is activated and division occurs is, however, not the same in all circumstances. The cell size necessary for division is modulated by growth conditions, such that cells in conditions low in nutrients will divide at a smaller size. This was shown to occur because these cells shorten the time until they divide rather than altering their growth rate (Fantes, P. and Nurse, P., 1977). The negative Cdc2 regulator *wee1*$^+$ was identified as a genetic element of this cell size control (Fantes, P.A. and Nurse, P., 1978), and since *wee1-50* mutants themselves had a critical size for DNA replication (Thuriaux, P. et al., 1978) it was assumed there were two size controls in fission yeast, at G1/S and G2/M (Figure 1.5). In *wee1* mutants the G2/M control was lost so daughter cells were subsequently too small to pass the G1/S control and they grow as small cells with 1C DNA content, but in wild type cells the G1/S size control was cryptic since they were always larger than the critical size needed to pass this control. Recent data has suggested that the Rum1 protein, which inhibits MPF during G1, may have some role in the G1/S size control, and this idea is supported by the fact that the long G1 period normally seen in *wee1-50* disappears in a *wee1-50 rum1Δ* double mutant (Moreno, S. et al., 1994; Moreno, S. and Nurse, P., 1994; Sveiczer, A. et al., 1996).

Control of cell growth

Thus careful genetic analysis in fission yeast has revealed that two size controls exist in the cell and if growth is inhibited these controls will act to increase the time between cell cycle transitions to maintain an optimum cell size for the growing conditions. However, it is still not known, firstly, how growth itself is switched on or off and secondly, how the growth conditions themselves are detected by, and modulate, elements of the cell size controls. The question of how cell growth is controlled may be even more relevant in

higher eukaryotes, where there is some evidence that certain types of cell may not have cell size checkpoints, but instead regulate cell growth and proliferation independently through extracellular signals (Conlon, I. and Raff, M., 2003; Conlon, I.J. et al., 2001; Edgar, B.A., 1999; Verdu, J. et al., 1999). If this is the case mechanisms must exist to switch cell growth on and off in response to nutrient availability and other extracellular signals. One proposed model is that protein synthesis rates may be critical determinants of cell growth. In yeast, genome-wide screens for cell size mutants have revealed many genes involved in ribosome biogenesis and protein translation govern cell growth (Jorgensen, P. et al., 2002) and in fact protein translation may also be the mechanism which links cell growth to the cell cycle, since translation of the mRNA of cell cycle regulators could be a means to arrest the cell cycle if growth is inhibited (Daga, R.R. and Jimenez, J., 1999; Polymenis, M. and Schmidt, E.V., 1997; Polymenis, M. and Schmidt, E.V., 1999). Protein synthesis is also a likely candidate mechanism by which cell growth is controlled in higher eukaryotes, since many growth regulatory pathways have been shown to regulate translation initiation and ribosome biogenesis. A good example of this kind of growth regulatory pathway is the target of rapamycin (TOR) pathway (Hay, N. and Sonenberg, N., 2004). TOR, an evolutionarily conserved protein kinase, is able to detect nutrient availability and growth factors such as insulin, and relay these signals to the translational machinery, particularly controlling whether ribosomes are initially recruited to the mRNA. The TOR pathway is likely to be a major regulator of cell growth in response to extracellular conditions in all eukaryotes. In addition there are other metabolic processes, such as nucleotide biosynthesis or mitochondrial function, which could also be targets for growth control mechanisms. How much modulation of these processes can affect cell growth and which signaling pathways can alter the rate of these processes remains to be determined.

1.8 Cell Stress

All cells must be able to respond to their environment and changing conditions. As described above, cells have developed the ability to arrest both the cell cycle and cell growth in response to nutrient deprivation or extracellular factors. In a similar way growth and division must also be arrested in the presence of toxic molecules or radiation which would cause damage to the cell. This arrest allows the cells to repair any damage so that optimal conditions for growth and division can be restored. The mechanisms by which cells are arrested in response to such stresses are known as checkpoints (Hartwell, L.H. and Weinert, T.A., 1989). Distinct classes of checkpoint are activated in response to different stresses and then, when the cell has recovered, these checkpoints are switched off and the cell cycle continues.

The heavy metal cadmium is a common environmental contaminant which most eukaryotic cells in their native surroundings will at some time be exposed to. Cadmium is extremely toxic to cells. It can react with thiol groups and displace metal ions from proteins, causing a multitude of physiological consequences. It also depletes glutathione from the cell, leading to oxidative stress (Stohs, S.J. and Bagchi, D., 1995). In addition it has been shown that cadmium can directly inhibit the mismatch repair (MMR) system leading to increased DNA mutation rates (Jin, Y.H. et al., 2003). Recent studies in budding and fission yeast have revealed the global changes in gene transcription which occur upon exposure to cadmium and revealed the mechanisms used to deal with such stress (Causton, H.C. et al., 2001; Chen, D. et al., 2003; Gasch, A.P. et al., 2000). These studies confirmed earlier work suggesting that the major method cells use to respond to cadmium is sequestration by glutathione, g-L-glutamyl-L-cysteinylglycine (GSH), which is subsequently exported into the vacuole (Li, Z.S. et al., 1997). GSH is a downstream product of the sulphate assimilation pathway, where inorganic

sulphur is assimilated from the environment into sulphur amino acids. In budding yeast most of the genes in this pathway are upregulated in response to cadmium (Fauchon, M. et al., 2002) and this is believed to be through the Met4 transcription factor (see above and Wheeler, G.L. et al., 2002). In fission yeast only a select group of these genes are induced, mainly sulphate transporter genes responsible for the initial step of sulphur uptake (Chen, D. et al., 2003).

How the cells respond to cadmium stress in terms of cell growth or division is less well understood. It would obviously be sensible for cells to arrest these processes in the presence of such a toxic compound but there has been little research into whether this is the case and if so what the regulators of this response are. There are no obvious cell cycle regulators whose expression changes in response specifically to cadmium, but many genes found to be regulated in other types of stress, known as the core environmental stress response (CESR), have altered expression in response to cadmium (Chen, D. et al., 2003). Many of these genes are predicted to function in carbohydrate metabolism, protein synthesis and other metabolic functions. It has been suggested that stressed cells reprogram such metabolic functions to limit cell growth and direct energy to the synthesis of stress-protective molecules. If this is the case it is feasible that some stress checkpoints, such as cadmium, arrest cells through cell growth as opposed to working through the cell cycle regulators.

In this thesis I will show that the SCF complex is involved in the link between cadmium stress and cell growth. Mutants in a fission yeast F-box protein show an overactive transcriptional response to cadmium and appear to arrest cell growth as a result. Thus this suggests a link between ubiquitin-mediated proteolysis, cadmium stress and cell growth arrest.

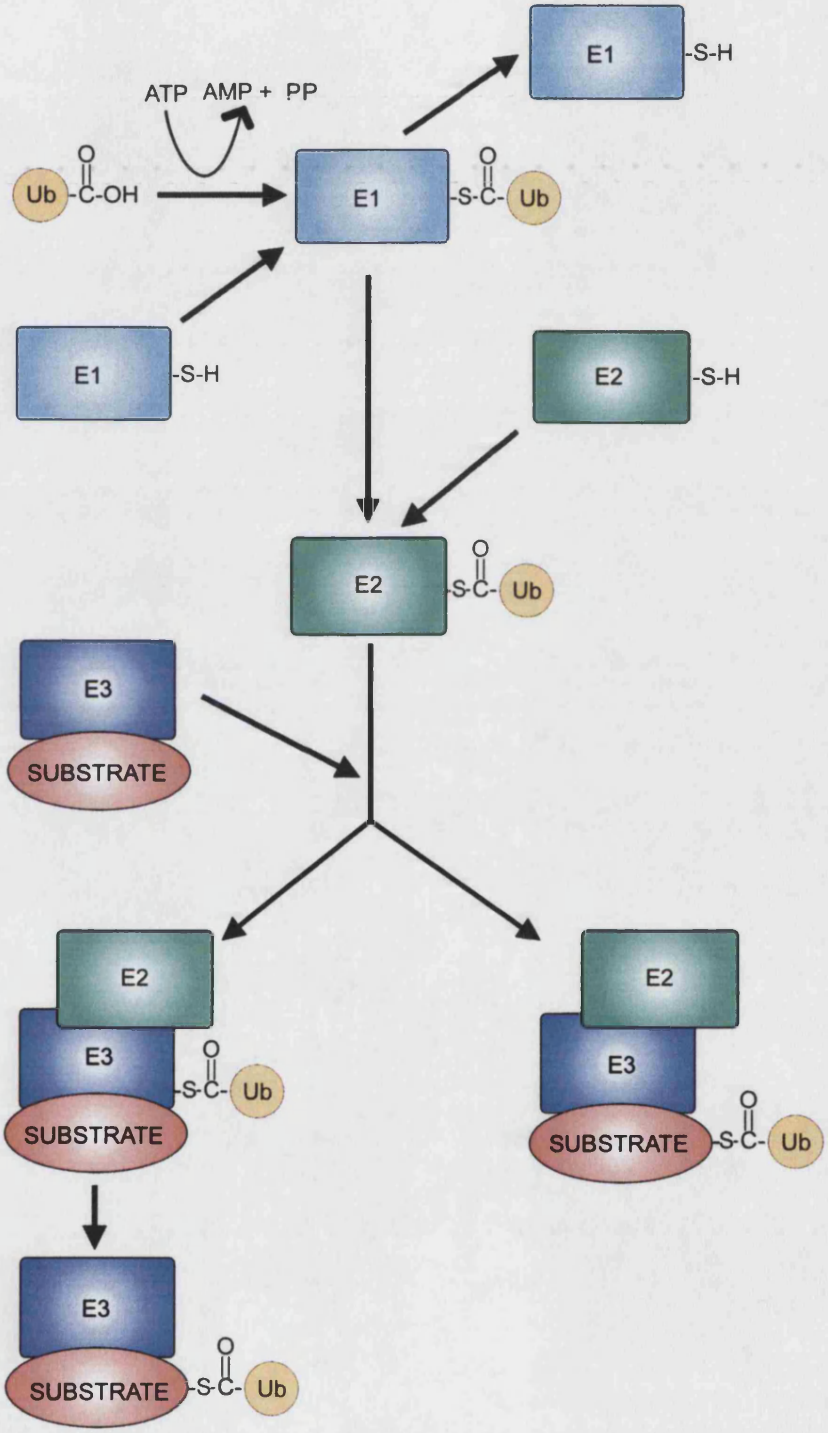


Figure 1.1 The ubiquitin conjugation pathway.
Ubiquitylation occurs through sequential steps, catalysed by activating (E1), conjugating (E2) and ligase (E3) enzymes. For simplicity the initial formation of an ubiquitin adenylate intermediate in the E1 reaction is not shown.

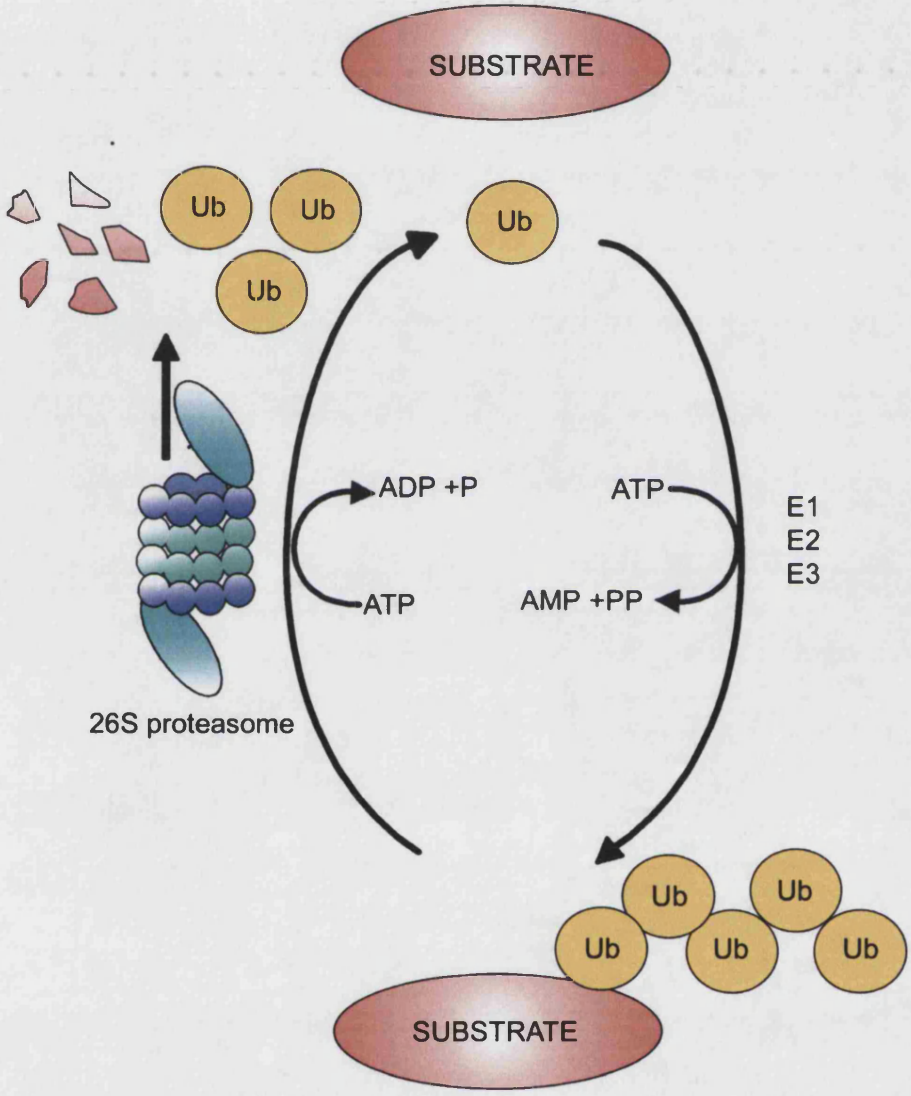


Figure 1.2 An overview of the ubiquitin/proteasome pathway.
ATP is used in conjugative (right) and degradative (left) phases. Following degradation by the proteasome ubiquitin can be recycled.

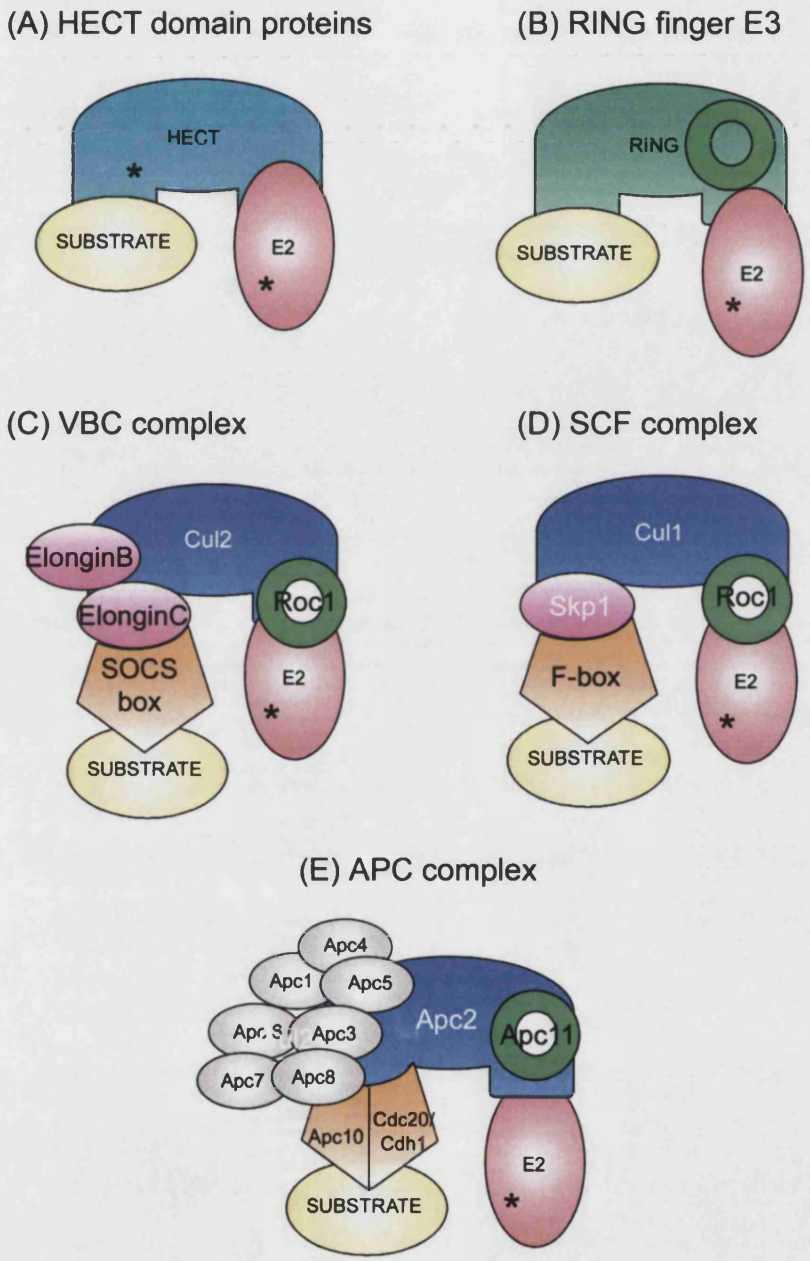


Figure 1.3 The known families of E3 ubiquitin ligases.
The majority of known ubiquitin ligases can be categorised as containing a HECT (light blue) or RING finger(green) domain protein. The multisubunit E3s identified so far contain a cullin protein (dark blue) and at least one substrate adaptor protein (orange). Proteins homologous to Skp1 are shown in pink and the E2 enzyme in red. Acceptor sites for thioubiquitin conjugates are shown by an asterisk. All diagrams are models , they do not illustrate actual structure of complexes.

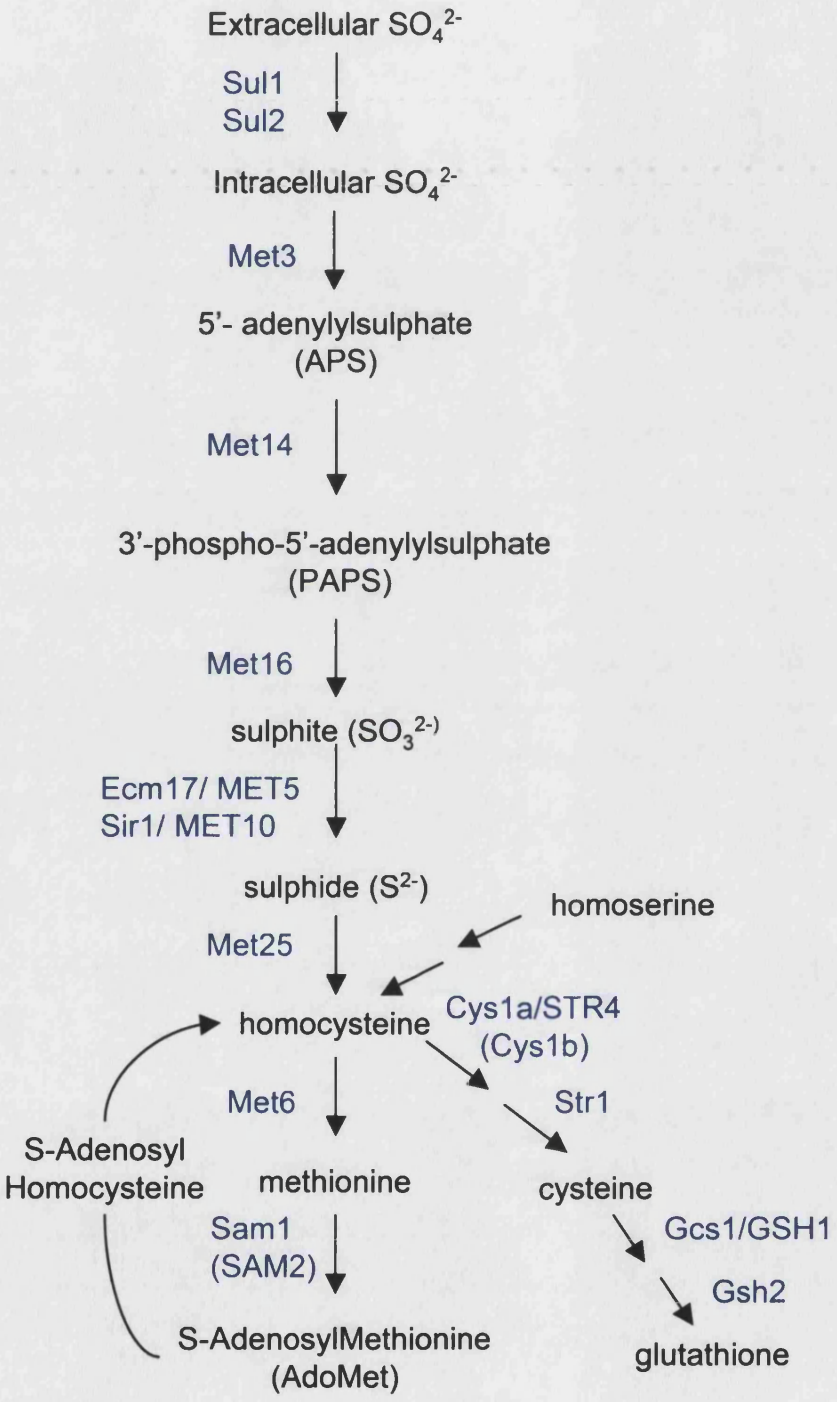


Figure 1.4 The sulphate assimilation pathway in fission and budding yeast

Enzymes responsible for each step of pathway are shown in blue, products in black. Where there are different names for enzymes the budding yeast name is shown in upper case and brackets indicate no homologue. For simplicity not all intermediates or enzymes are shown.

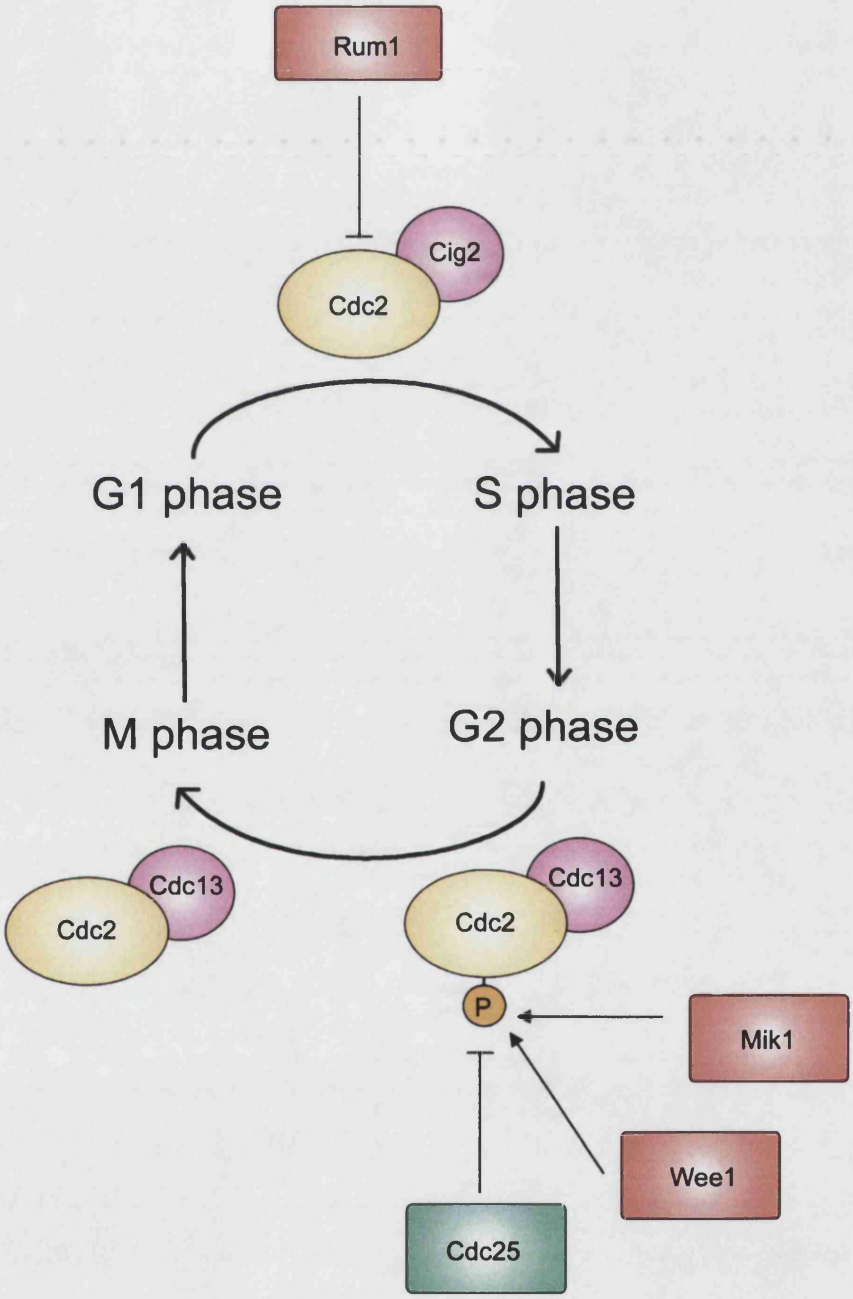


Figure 1.5 The two size control points in the fission yeast cell cycle.

The G2/M checkpoint is controlled through Wee1 kinase which maintains an inhibitory Tyr15 phosphorylation on Cdc2 until an appropriate cell size is reached. The G1/S checkpoint is cryptic, since cells are usually large enough to pass this point following cytokinesis in the previous cycle. It has been suggested that this checkpoint acts through the Cdc2 inhibitor Rum1

Table 1 Selected HECT domain ubiquitin ligases and their substrates (based on Pickart 2001)

Organism	E3 ligase	Substrate	Function
S. cerevisiae	Rsp5	Rbp1, RNA pol II LS	Rbp1 degradation after DNA damage
		Plasma membrane permeases/ receptors	Receptor endocytosis
		Spt23, Mga2 transcription factors	Leads to processing and activation of Spt23/Mga2
	Tom1	Spt7 transcription factor	Regulates transcriptional activation.
H. sapiens (and other higher organisms)	E6-AP	p53 in HPV infected cells	Downregulation of p53 by virus.
		Src family tyrosine kinases	Degradation of activated kinases
		HHR23A nucleotide excision repair factors	Degradation during S-phase
		Mcm7, replication licensing factor subunit	Degradation of Mcm7, enhanced in HPV infected cells.
	Nedd4	Rbp1, PNA pol II LS	Rbp1 degradation.
		ENaC, sodium channel	Targets ENaC for endocytosis regulating numbers of channel molecules
	Smurf1	Smad proteins	Regulates TGF-beta signaling output
	Itch	Notch	Regulation of cell fate decision/ immune responses

Table 2 Selected single subunit RING finger ubiquitin ligases and their substrates (based on Pickart 2001)

Organism	E3 ligase	Substrate	Function
S. cerevisiae	Ubr1	Substrates of N-end rule pathway, e.g.. Cup9, required for peptide import	
	Rad18	PCNA	Monoubiquitylation of PCNA is induced by DNA damage and needed for damage response
	Rad5	Unknown but...	Required for Rad18 dependant PCNA monoubiquitylation
H. sapiens (and other higher organisms)	E3α/Ubr1	Encephalomyocarditis virus 3C protease	EMC 3C protease degradation
	Rad18	PCNA	Probably the same function as in yeast
	c-Cbl	epidermal growth factor receptor (EGFR) and platelet derived growth factor (PDGF) receptor	Downregulation of activated receptor protein tyrosine kinases
	Mdm2	p53	Modulates p53 turnover, inhibited in respose to DNA damage leading to increased p53 transcription.
	Siah-1	Deleted in colorectal Cancer (DCC)	DCC degradation
		T-STAR, RNA binding Protein	Regulation of alternative splicing mechanisms
	Inhibitors of Apoptosis (IAPs)	Apoptosis regulators e.g. caspases, second mitochondria-derived activator of caspases (Smac)/DIABLO	Regulate apoptosis

Table 3 List of fission yeast F-box proteins

F-box protein	Amino acids	Associated motifs	Homologues *S.cerevisiae*	Homologues *H.sapiens*	Deletion phenotype	Derivation
Pop1	775	WD40	Cdc4	Fbw7	Polyploid	(Kominami and Toda,1997)
Pop2	703	WD40	Cdc4	Fbw7	Polyploid	(Kominami *et al.* 1998)
Pof1	605	WD40	Met30	β-TRCP	Essential	(Lehmann *et al.* 2004)
Pof2	463	LRR	Grr1	?	None	(Lehmann *et al.* 2004)
Pof3	577	TPR/LRR	Dia2	?	G2 delay	(Katayama *et al.* 2002)
Pof4	199	None found	Ela1	Elongin A	None	(Lehmann *et al.* 2004)
Pof5	348	None found	Ydr360c	?	None	(Lehmann *et al.* 2004)
Pof6	872	CAAX	Rcy1	?	Essential	(Lehmann *et al.* 2004)
Pof7	361	None found	Hrt3	?	None	(Lehmann *et al.* 2004)
Pof8	402	None found	Ufo1	?	None	(Lehmann *et al.* 2004)
Pof9	467	None found	Ybr280c	Fbx9	None	(Lehmann *et al.* 2004)
Pof10	662	WD40	Yml088w	?	None	(Lehmann *et al.* 2004)
Pof11	506	WD40	?	β-TRCP1	None	(Lehmann *et al.* 2004)
Pof12	440	None found	?	?	None	(Lehmann *et al.* 2004)
Pof13	396	None found	?	?	None	(Lehmann *et al.* 2004)
Fdh1	878	UvrD/REP helicase	?	Fbh1	G2 delay	(Lehmann *et al.* 2004)

Chapter 2

Initial Characterisation of Pof1

Introduction

This chapter describes the initial characterisation of the *S. pombe* F-box protein Pof1. Pof1 was identified in an earlier study using sequence homology searches to find all F-box motif containing proteins in fission yeast. Deletion of the genes coding for these proteins revealed only two were essential for fission yeast cell viability, one of which was *pof1*$^{+}$. Thus Pof1 characterisation would reveal one of the essential functions of the *S. pombe* SCF complex. The starting point for this work was to confirm the essentiality of *pof1*$^{+}$ and attempt to look at the phenotype of cells with disabled Pof1 function. In addition the localisation of Pof1 was examined in the hope that this would present some idea as to its function.

2.1 Deletion of All S. pombe F-box Proteins

In order to identify *S. pombe* F-box proteins which are essential to the cell, and therefore may be responsible for recognising some essential SCF substrate, all 14 genes encoding novel F-box proteins were deleted in a lab-wide effort (Lehmann, A. et al., 2004) (Pop1 and Pop2 were already characterised). This was carried out using a gene-replacement method.

Homology searches for F-box proteins

The F-box proteins had been first identified using sequence homology searches. The F-box protein Pop1 had been first identified in a screen for polyploid mutants. The sequence of the Pop1 F-box was then used to do BLASTP searches on The Wellcome Trust Sanger Centre *S. pombe* GeneDB server. This identified Pop2 and Pof1-8. Identification of the other F-box proteins came later following the discovery of a novel *S. cerevisiae* F-box protein, YBR280c. The YBR280c sequence was used to do further BLASTP searches on the Sanger Centre GeneDB server. This pulled out Pof9 and Pof10. The Pof10 sequence was then used itself to do further BLASTP searches and identified Pof11, 12 and 13. Thus using sequential rounds of database searches 13 novel proteins containing the F-box motif were identified.

The gene replacement method

The gene replacement method is a Polymerase Chain Reaction (PCR) based method which involves the amplification of a marker gene sequence using primers flanked on either side by approximately 80 base pairs of DNA sequence homologous to those flanking the gene of interest (Bahler, J. et al., 1998). Transformation of *S. pombe* cells with this PCR product leads to

recombination of the fragment into the targeted locus (as illustrated in Figure 2.3 where the deletion of *pof1*$^+$ using this method is described). Cells containing integrated products can be selected by the marker gene, in this case either *ura*$^+$, which allows growth on uracil-free media, or kanamycin resistance (*kan*^{R}), which allows growth on media containing the antibiotic G418. Because it was not known before deletion if these genes would be essential to growth the deletions were initially constructed in a diploid *S. pombe* strain. *S. pombe* is a naturally haploid organism but can be maintained as a diploid using a complementation technique. Haploid cells containing a mutation in the *ade6* gene grow as red colonies on low-adenine media. If two haploid strains with different, but complementary, *ade6* mutant alleles are crossed the resulting diploid can grow as white colonies on adenine-free media. It is known that two adenine mutant alleles, *ade6-M210* and *ade6-M216*, can display such complementation. Thus these strains were crossed and an Ade$^+$ diploid selected. These diploid cells were used for transformation with the F-box deletion products. If the F-box protein was essential the cells could still grow as they would be heterozygous for the deletion. The diploids could be selected on uracil-free or G418-containing media and correct insertion of the PCR fragment confirmed by colony PCR, where a PCR reaction is carried out using genomic DNA as a template and a primer pair where one primer binds within the marker gene ORF and the other just outside the targeted gene. Only cells containing correctly inserted marker gene can produce a PCR product and these cells were then sporulated and subjected to tetrad dissection to isolate haploids containing the F-box gene deletion. If haploids containing the deletion marker gene did not grow it was assumed the F-box protein was essential. Using this method all 14 F-box containing genes were deleted. The phenotypes of cells containing these deletions are shown in Table 3, Chapter1.

Only two *S. pombe* F-box proteins are essential

Only two of these fourteen novel F-box proteins were found to be essential for cell growth, meaning no spores containing the marker for these deletions could ever form viable colonies following tetrad dissection onto rich media at 26°C. These F-box proteins were Pof1 and Pof6 (Hermand, D. et al., 2003). It was reasoned that these proteins must recruit some substrate to the SCF complex which is lethal to the cell if allowed to accumulate. Thus the degradation of these substrates represents the essential function of the SCF in *S. pombe*. I decided to characterise the role of Pof1 in the cell in order to gain some insight into why the SCF complex is essential in fission yeast.

2.2 The Pof1 F-box protein

Pof1 is a 605 amino acid protein. It is most homologous to budding yeast F-box protein Met30 and like Met30 it contains an N-terminal F-box motif and seven C-terminal WD40 repeat domains (van der Voorn, L. and Ploegh, H.L., 1992), as illustrated in Figure 2.1. F-box motifs are relatively variable but the Pof1 residues Leu^{113}, Pro^{114}, Iso^{121}, and Leu^{125} are homologous to residues found to be the least variable amongst F-box proteins (Kipreos, E.T. and Pagano, M., 2000), suggesting Pof1 has a relatively well conserved F-box motif.

Pof1 has been shown to interact with other SCF components Skp1 (Lehmann, A. et al., 2004) and Cullin1 (Figure 2.2, data is shown for information purposes but experiment was carried out by S. Katayama), suggesting it is able to interact with components of the core SCF complex.

2.3 Pof1 is essential for spore germination and vegetative growth

Pof1 is essential for spore germination

In order to confirm that Pof1 was essential and look at the phenotype of cells that died due to lack of Pof1 function, strains containing a *pof1*$^+$ deletion were freshly constructed (this was necessary as the original strains failed to sporulate following recovery from storage conditions). This was done using the gene replacement method in a diploid strain as described above. Sequences and location of primers used can be found in Appendix A. Note that the disruption fragment does not overlap with genes upstream or downstream of *pof1*$^+$. A *ura4*$^+$ marker was used to replace the *pof1*$^+$ gene and transformants selected using plates lacking uracil. Eighteen colonies could grow on these plates, but following microscopic examination only five appeared to be larger, so presumably diploid, cells. These five colonies were patched onto fresh plates lacking uracil. They were then tested for correct incorporation of the *ura4*$^+$ fragment using colony PCR as described earlier. One colony was positive for the correctly integrated fragment. This ura$^+$ diploid was induced to sporulate on plates low in nitrogen, and the resulting tetrads were dissected onto YE5S plates. After approximately 5 days at 26°C colonies could be seen growing in a 2:0 pattern (Figure 2.4A), thus only 50% of spores from the diploid were viable. Replica plating of these colonies onto plates lacking uracil confirmed all viable haploid cells were lacking the *ura4*$^+$ gene (data not shown), thus confirming that deletion of *pof1*$^+$ was lethal.

In order to look at the phenotype of these spores lacking *pof1*$^+$ they were next germinated in liquid media. The *ura4*$^+$ containing diploids were sporulated in low nitrogen liquid media, then this media filtered away and replaced with selective media. Half of the spores went into media containing uracil, in which all spores should be able to germinate and grow, the other half went into

uracil-free media, which should allow only spores containing the *pof1*$^+$ deletion to develop. This method allows a much larger number of *pof1*$^+$-deleted cells to be observed than tetrad analysis. The cells grown in uracil-containing media looked wild type although a small number of non-germinated spores could be observed (Figure 2.4B, left-hand side). The cells grown in uracil-free media however, had mostly failed to germinate and those that had were extremely small (Figure 2.4B, right-hand side). Observation of *pof1*$^+$-deleted cells dissected by tetrad analysis on plates also supported a failure in germination in *pof1*$^+$-deleted spores (data not shown).

Pof1 is essential for vegetative growth

A large number of spores containing the *pof1*$^+$ deletion appeared unable to germinate. This suggested that Pof1 might have some role in the germination process. In order to confirm that it also was essential for vegetative growth a plasmid dependency test was carried out. The *pof1*$^+$ ORF was subcloned into a multicopy plasmid, pREP1, under the control of a thiamine repressible promoter (Maundrell, K., 1990). The plasmid contained a selectable *LEU2* marker gene which allows growth on media lacking leucine. The *pof1*$^+$/*pof1*$^+$::*ura4*$^+$ heterozygous diploid already contained a homozygous mutation in the *leu1*$^+$ locus (which can be complemented by *LEU2)*, so could be transformed with the *pof1*$^+$ plasmid and leu$^+$ transformants selected on leucine-free media. At the same time, as a control, the *pof1*$^+$/*pof1*$^+$::*ura4* diploid was transformed with an empty pREP1 plasmid and again leu$^+$ transformants selected. Diploids containing pREP1-*pof1*$^+$ and empty pREP1 were allowed to sporulate and spores spread onto plates lacking uracil-only, leucine-only and *containing* both uracil and leucine. This method is known as random spore analysis. The following numbers of colonies grew on plates after four days at 26°C:

pREP1-*pof1*$^+$: minus uracil- 18

minus leucine- 25

containing both- 153

pREP1 empty: minus uracil- 51

minus leucine- 96

containing both- 206

Only cells transformed with pREP1-*pof1*$^+$ produced a significant number of viable colonies on plates lacking uracil, suggesting that the *pof1*$^+$ plasmid was able to compensate for the *pof1*$^+$*::ura4*$^+$ deletion. In order to confirm that these cells really contained a *pof1*$^+$ deletion and were not just heterozygous diploid, I selected six ura$^+$, leu$^+$ colonies and patched them onto plates lacking adenine. As described above the diploid cells used for this experiment contained complementary mutations in the adenine gene, *ade6*$^+$, thus could grow white without an external adenine source, but haploid cells cannot. Four of the six colonies selected could not grow on adenine-free plates, confirming their haploid status. The deletion of *pof1*$^+$ by *ura4*$^+$ was next confirmed using colony PCR as described above. Thus I was confident that these four strains were all haploid cells containing a *pof1*$^+$ deletion and a *pof1*$^+$ plasmid. Plasmid dependency was now tested to see if *pof1*$^+$ was necessary for vegetative growth of cells. If it were not, *ura4*$^+$ containing cells which had lost the plasmid, so could not grow without a leucine source, should be recoverable. Cells from the four independent transformants were grown in rich media overnight, a condition where they are likely to lose multicopy plasmids. Approximately 10^4 cells of each strain were then plated onto rich medium plates and after colonies appeared these were replica plated onto plates lacking uracil, leucine and uracil and leucine. All of the colonies which grew without uracil could grow without leucine, none could be found which contained the *ura4*$^+$ gene but had lost the plasmid. This confirmed that *pof1*$^+$ was necessary for vegetative growth.

2.4 Pof1 localises to the nucleus throughout the cell cycle

Tagging the Pof1 protein

The Pof1 protein was tagged with a GFP protein epitope. This was achieved by tagging the C-terminus of the *pof1*$^{+}$ gene with GFP and a kanamycin resistance gene which provides a selectable marker. Using a similar method to that used for gene deletion (Bahler, J. et al., 1998), a GFP-kanamycin cassette was amplified using PCR. The primers used for this PCR again had flanking DNA sequence homologous to the target gene sequences, in the case of C-terminal tagging this must be homologous to sequences downstream of the STOP codon. Wild type haploid cells were transformed with this PCR product, resulting in homologous integration of the GFP-kanamycin sequence, as shown in Figure 2.5. Seven transformants were selected by their ability to grow on plates containing antibiotic G418, and correct integration of the GFP-kanamycin sequence confirmed by colony PCR, resulting in two positive candidates. Western blot analysis using anti-GFP antibodies was performed to confirm that the tagged Pof1 protein was being correctly expressed. GFP tagging of the Pof1 protein did not appear to interfere with Pof1 function as the strain appeared as wild type.

Pof1 is a nuclear protein

Because chemically fixing cells can sometimes alter protein localisation Pof1-GFP cells were observed live, after growing in liquid media overnight. Pof1-GFP appeared to localise to the nuclear region. In order to look at this more carefully Pof1-GFP cells were stained with Hoechst 33342, a DNA–binding stain which has the advantage of working on non-permealised, live cells (Chikashige, Y. et al., 1994). Pof1-GFP and DNA staining were then observed. Pof1-GFP co-localised with the DNA staining (Figure 2.4A), suggesting that Pof1 is a nuclear protein. More detailed analysis showed that this appeared to be the case throughout the cell cycle, both in interphase and

mitotic cells (Figure 2.4B) During interphase Hoechst staining showed DNA as a characteristic semi-spherical shape. Pof1-GFP appeared to almost completely overlap with this signal suggesting that Pof1 associates with the DNA region of the nucleus in interphase (Figure 2.4B, 1 and 2). During mitosis Pof1-GFP also overlapped with the DNA signal, although in early anaphase there seemed to be a significant amount of Pof1-GFP in the region between the dividing DNA (Figure 2.4B, 3), suggesting that Pof1 can also localise to other regions of the nucleus.

Summary

Pof1 is an essential fission yeast F-box protein. The majority of spores deleted for *pof1*$^{+}$cannot germinate and those that do fail to grow or divide. Cells deleted for *pof1*$^{+}$ can only grow if carrying a *pof1*$^{+}$ plasmid. Thus Pof1 is essential for germination and vegetative growth. Pof1 is a nuclear protein throughout the cell cycle and during interphase. It appears to associate with the DNA-containing region of the nucleus

Discussion

The deletion of *pof1*$^{+}$ in diploid cells confirmed that it is indeed essential. Many spores containing the deletion failed to germinate and those that did appeared small and, since colonies failed to form, were obviously not competent for cell division. This suggested that Pof1 function was required for some early essential function making viable cells from spores. Germination involves a dormant *S. pombe* spore (said to be in G0) resuming mitotic division. This must only happen when nutrient conditions are favourable for cell growth. The genes and pathways responsible for germination remain largely unknown because of the difficulties in recovering mutants specifically deficient in these processes, however some ideas of these pathways have been revealed by recent genome wide screens for germination-defective

mutants in budding yeast (Deutschbauer, A.M. et al., 2002). From these experiments it appears germination defects can result from several different types of genes. Firstly, meiotic mutants often form spores lacking the correct chromosome complement, thus which are unable to germinate. Secondly, a class of mutations in genes specifically involved in the germination process exists and finally there are genes which are involved in a wider range of processes than just germination, such as nutrient sensing, cell growth and metabolism. The fact that *pof1*$^{+}$ is essential for vegetative growth suggests that it falls into this latter category. Pof1 function appears likely for some process important for cell growth, this could be either a fundamental process such as protein translation or a regulatory process such as nutrient sensing. One of the few germination defective mutants to be characterised in *S. pombe* is *mip1* (Shinozaki-Yabana, S. et al., 2000). This gene is essential for spore viability, with only 40% of *mip1*$^{+}$-deleted spores germinating. Mip1 has been shown to be required for mitotic cell growth as well as meiotic development. The phenotype of *pof1* cells is remarkably similar to *mip1*, with both showing a similar low germination efficiency, cells which do germinate being small and both being necessary for vegetative growth. This suggests that they may both fall into a similar class of growth regulatory genes.

The localisation of Pof1 to the nucleus throughout the cell cycle suggests that Pof1 has some role within the nucleus. Since Pof1 is an F-box protein and known to interact with other components of the SCF it is likely that this role involves substrate ubiquitylation. Many data in yeast suggest F-box proteins may have a specific subcellular location and that this may allow compartmentalised protein degradation. Far1, a yeast protein required to arrest the cell cycle and polarise the actin cytoskeleton during mating, is a known target of SCF^{Cdc4}(Henchoz, S. et al., 1997). It has been shown that Far1 degradation is dependant upon the localisation of Cdc4, with the stability of nuclear or cytoplasmic Far1 being dependant upon whether Cdc4 is

nuclear or cytoplasmic (Blondel, M. et al., 2000). Thus the nuclear localisation of Pof1 would suggest it is ubiquitylating some substrate in the nucleus and this could represent some spatial form of control over Pof1 function.

The yeast homologue of Pof1 is Met30. The SCF^{Met30} regulates the transcription factor Met4 (Patton, E.E. et al., 2000; Rouillon, A. et al., 2000) and thus, presumably because Met4 must be regulated in the nucleus, Met30 is nuclear. The nuclear localisation of Pof1 supports it having functional homology with Met30. Met30 lacks a definable nuclear localisation sequence but the amino- terminal half of Met30, including the F-box motif, has been shown to mediate Met30 nuclear import and retention (Brunson, L.E. et al., 2004). Examination of the Pof1 amino acid sequence suggests it also lacks an obvious definable nuclear localisation signal, thus it is possible this mechanism of nuclear import using sequences throughout the entire amino-terminal half of the protein is conserved between Met30 and Pof1.

A final interesting point to note from the Pof1 localisation data is that Pof1 appears to be constitutively expressed within the cell, with all cells appearing to show a Pof1-GFP signal. The abundance of some F-box proteins is known to be regulated during the cell cycle, but Pof1 seems to be always present in normally growing cells. Since many SCF substrates are known to require phosphorylation before they can be recruited by an F-box protein, the constitutive expression of Pof1 does not mean it is constitutively active, as this could be regulated at the level of the substrate specific kinase. However the fact that Pof1 is always expressed in cells could suggest that it must always be available to degrade its substrate, possibly in response to some commonly encountered condition such as nutrient availability changes or cell stress.

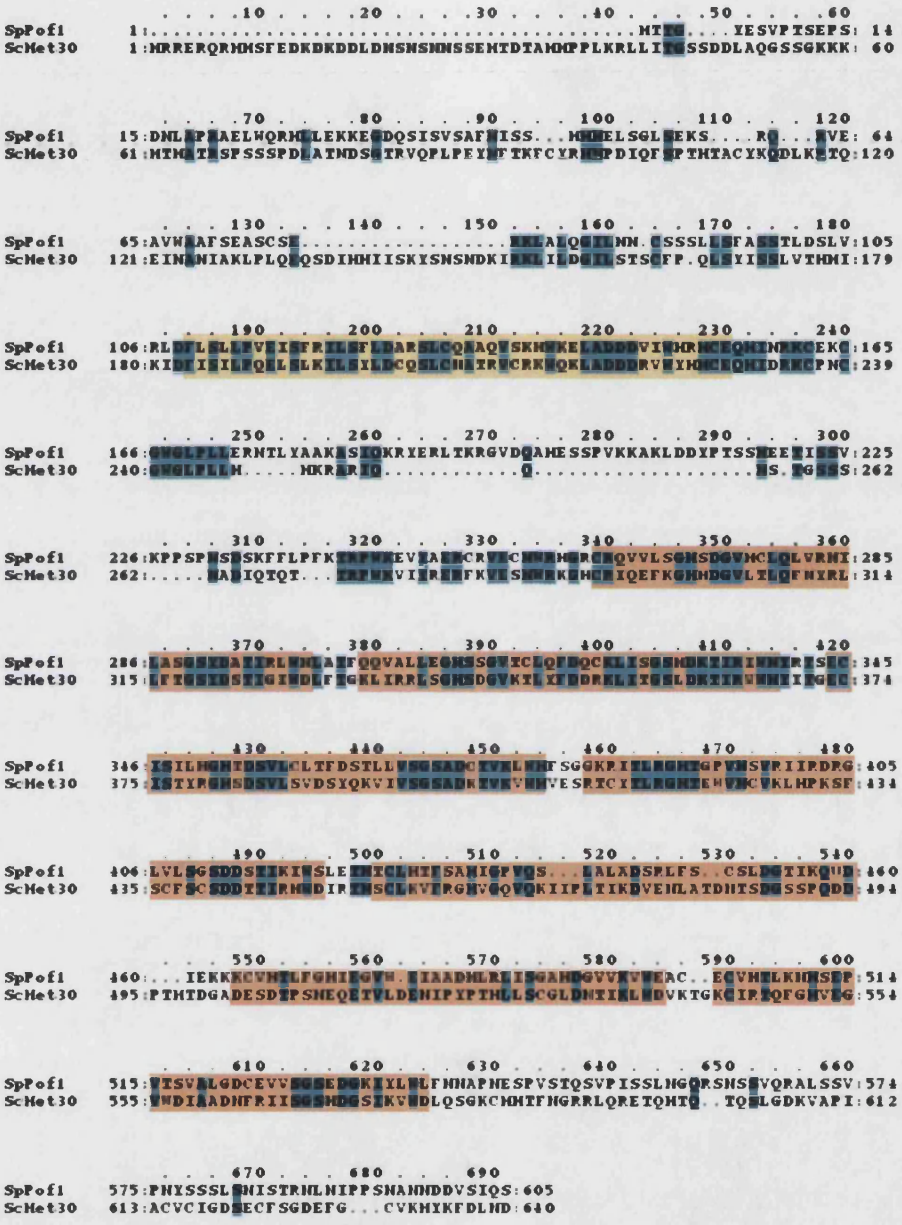


Figure 2.1 Sequence alignment of fission yeast F-box protein Pof1 and budding yeast homologue Met30.

For simplicity only identical residues are highlighted in green. The F-box motif is highlighted in yellow and sequences with high consensus to WD repeat motif are highlighted in orange.

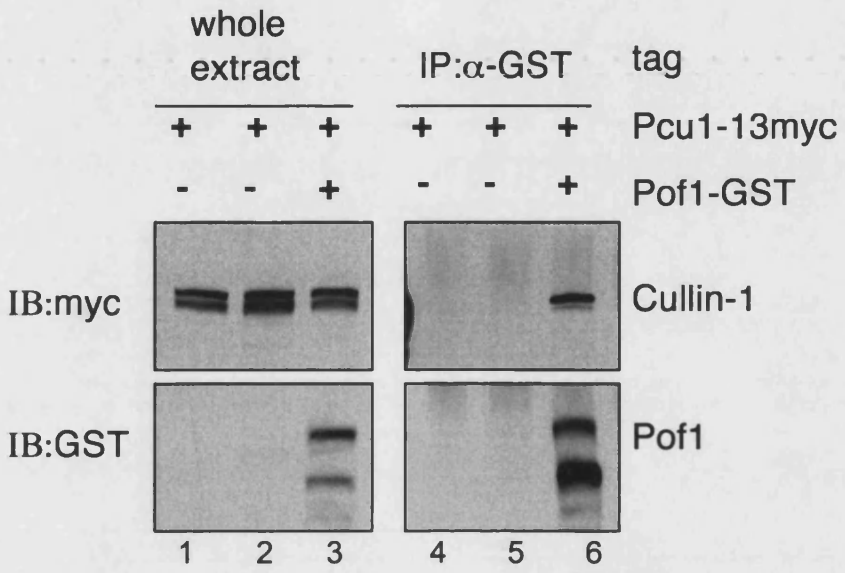


Figure 2.2 Interaction between Pof1 and cullin-1.
Protein extracts were prepared from a single tagged (*pcu1⁺-13myc*, lanes 1,2, 4 and 5), or double tagged strain (*pof1⁺-GST pcu1⁺-13myc*, lanes 3 and 6), and immunoprecipitation was performed with anti-GST (lanes 4 to 6). After running on SDS-PAGE, immunoblotting was performed with anti-Myc (upper) or anti-GST antibody (lower). Experiment carried out by S.Katayama (Harrison, Katayama, et al. 2004).

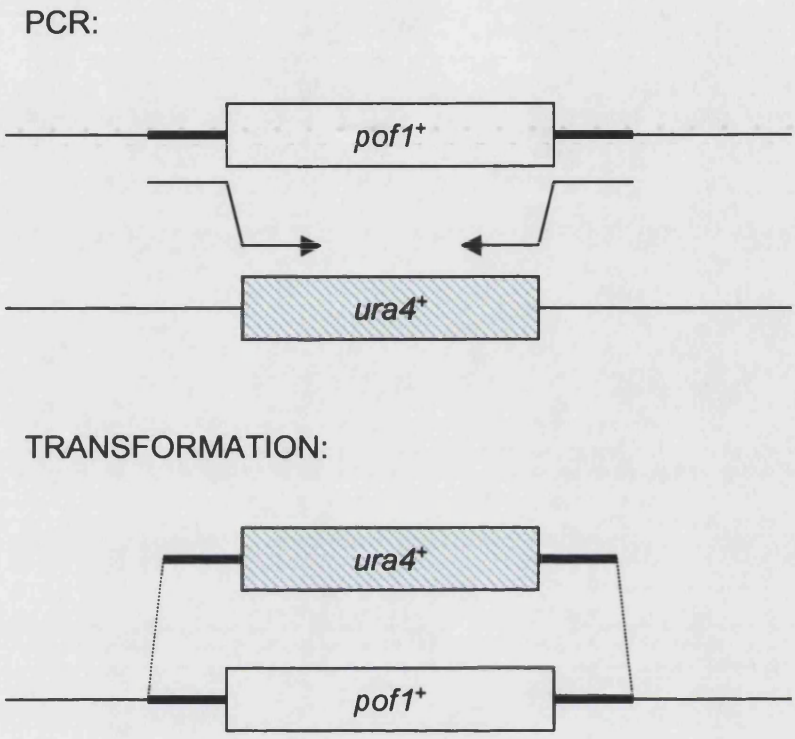


Figure 2.3 Gene deletion in *S. pombe*.
Complete deletion of the *pof1*$^+$ ORF by the *ura4*$^+$ sequence was carried out using PCR-generated fragments. The *ura4*$^+$ gene is amplified by PCR using primers with 80 base pair sequence homology to regions flanking the *pof1*$^+$ gene (illustrated with thick black lines). These PCR amplified fragments are transformed into wild type cells. Homologous recombination ensures integration into the *pof1*$^+$ locus (Bahler, Wu et al. 1998).

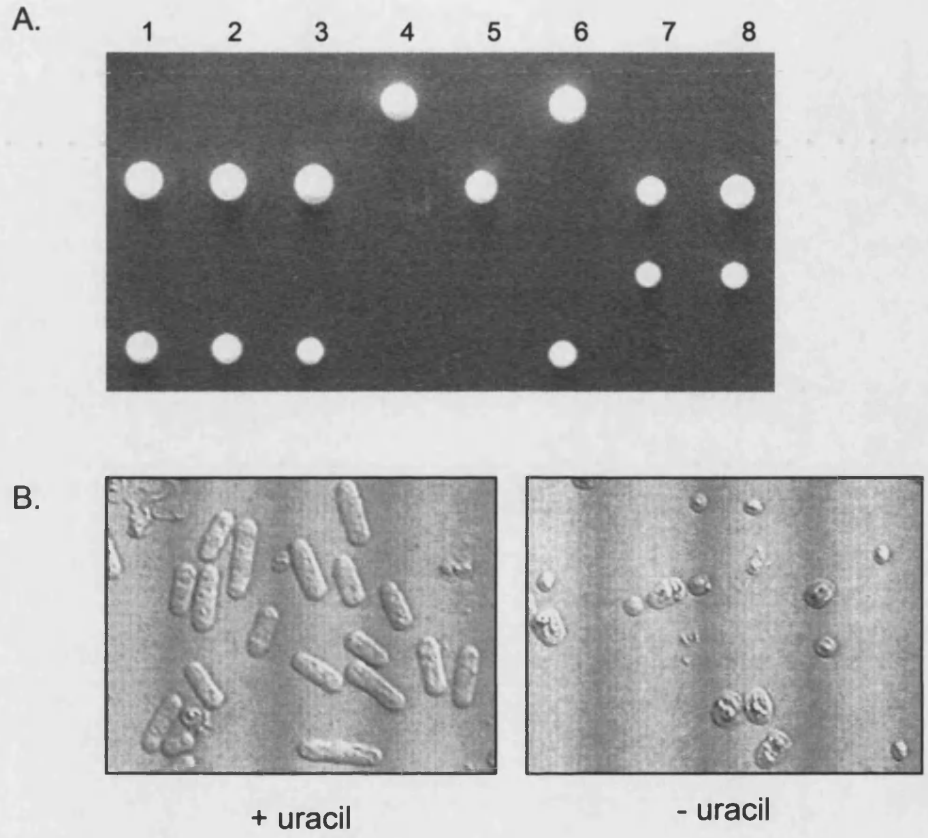


Figure 2.4 Deletion of *pof1*$^{+}$

A. Pof1 was deleted in a diploid strain using a *ura4*$^{+}$ cassete by the gene replacement method. *Ura4*$^{+}$ diploids were selected and after sporulation the resulting tetrads dissected onto YE5S and spores germinated at 26°C. Numbers 1-8 represent the individual diploids. **B.** The phenotype of cells lacking *pof1*$^{+}$. Spores from diploids heterozygous for a *pof1*$^{+}$::*ura4*$^{+}$ deletion were germinated in SD media either containing (lefthand side) or lacking (righthand side) uracil.

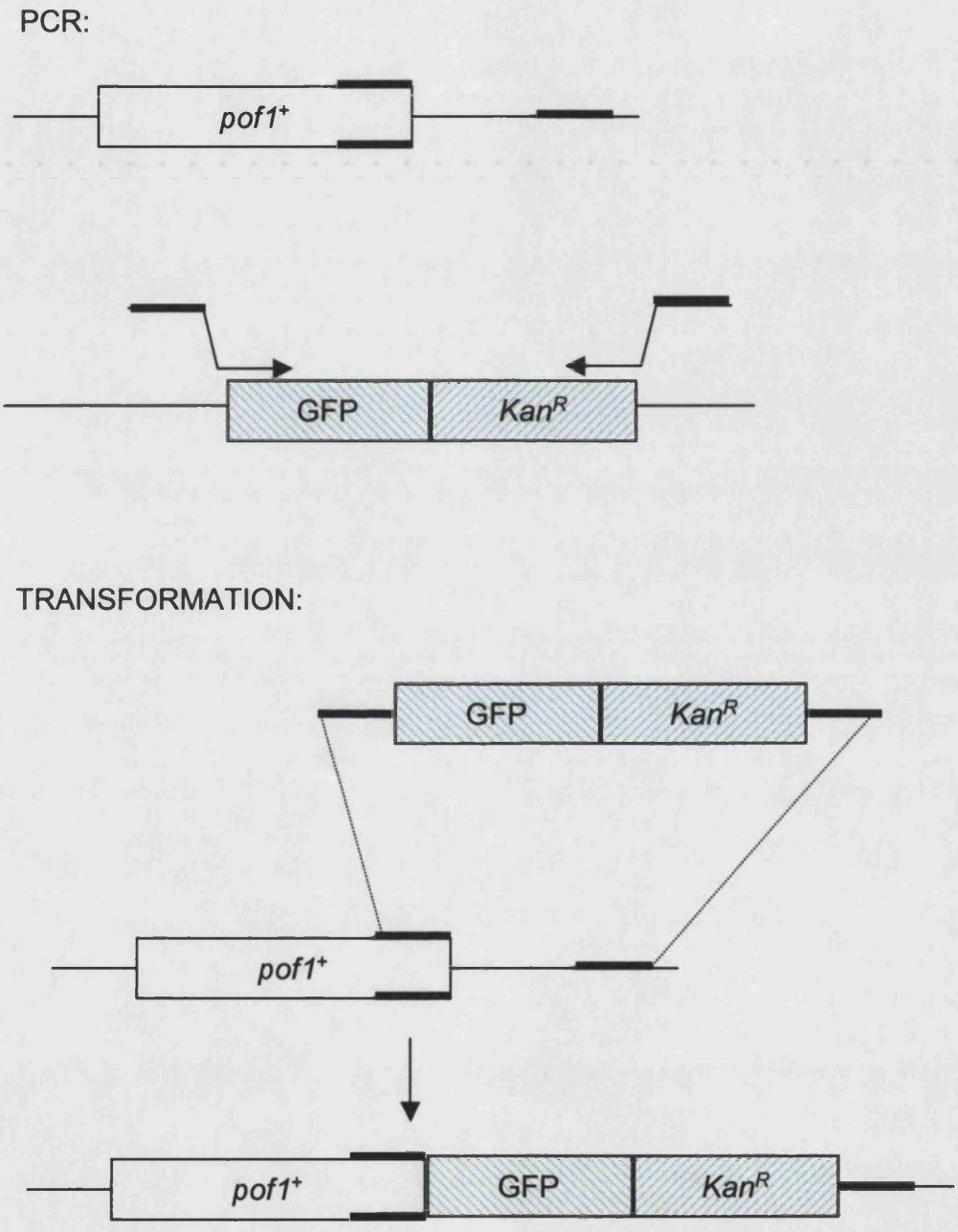


Figure 2.5 Gene tagging in *S. pombe*

C-terminal tagging with GFP epitopes was carried out using PCR-generated fragments. A construct containing the GFP tag and the kanamycin resistance gene as a marker is amplified by PCR using primers with 80 base pair sequence homology to regions C-terminal to the *pof1*$^{+}$ gene (illustrated with thick black lines). These PCR amplified fragments are transformed into wild type cells. Homologous recombination ensures integration into the *pof1*$^{+}$ locus (Bahler, Wu et al. 1998).

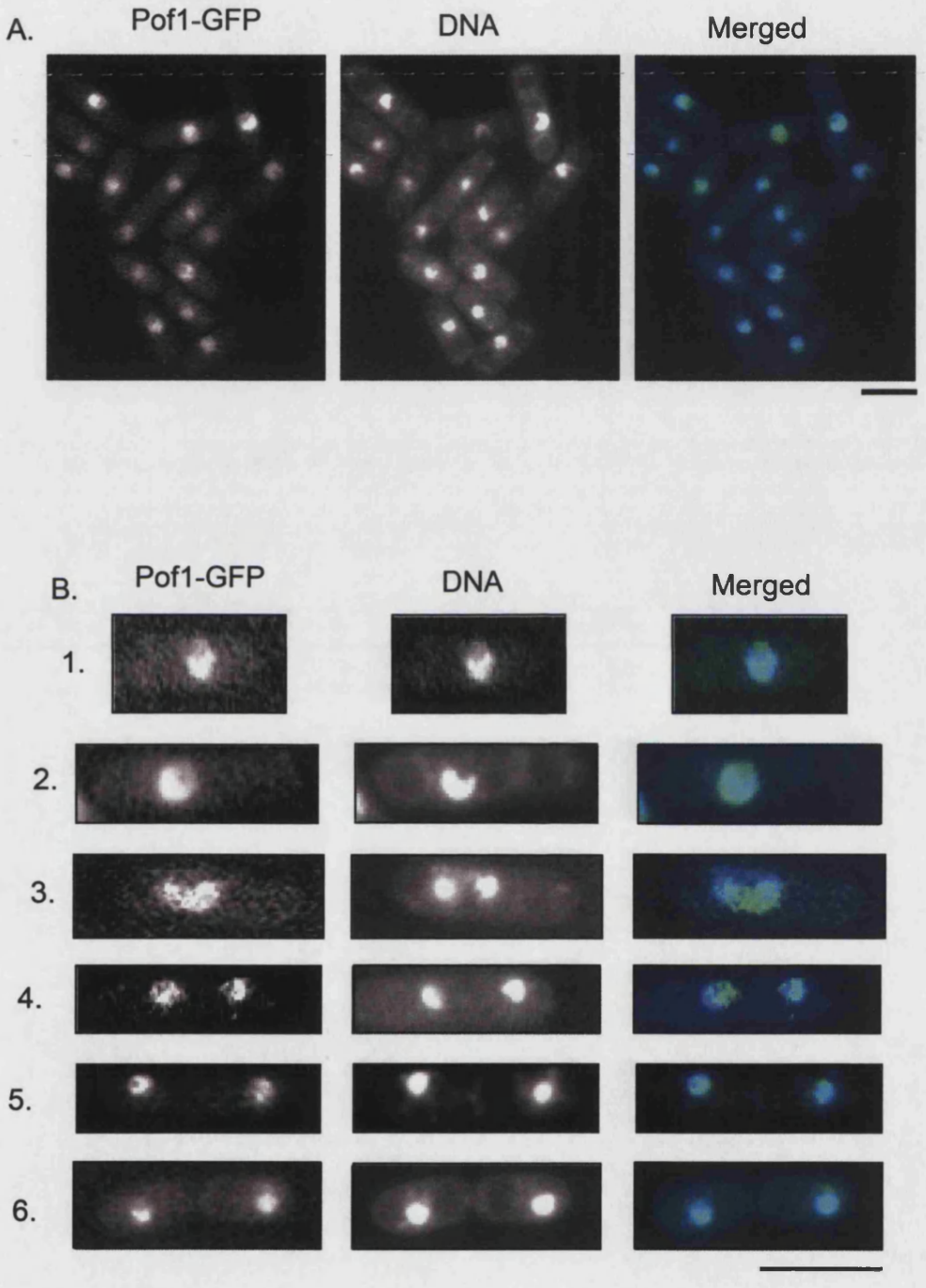


Figure 2.6 Localisation of Pof1

A. Fluorescence of Pof1-GFP in live cells is shown. **B.** Localisation of Pof1-GFP during the cell cycle. Representative images of interphase (1 and 2), early to late anaphase (3,4 and 5) and late cytokinesis (6) are shown. All cells were grown in rich media until exponential phase, stained with Hoechst and then live Pof1-GFP fluorescence observed. Bars indicate 10μM

Chapter 3

Isolation and characterisation of *pof1* temperature sensitive mutants

Introduction

The fact that *pof1*$^+$ is essential for cell viability hampered the characterisation of its function, since cells lacking the *pof1*$^+$ gene could obviously not be propagated. In order to examine the role of Pof1 further, temperature sensitive (ts) *pof1* alleles were isolated. This chapter describes the creation and isolation of these alleles and their subsequent phenotypic analysis. These cells could grow normally at 26°C but arrested as small cells at 36°C. A small cell phenotype can be caused by defects in cell cycle regulation or cell growth. The genetic interactions of *pof1* with cell cycle regulators were investigated and the progression of *pof1* mutants through the cell cycle following various synchronisation experiments was followed. This chapter describes these experiments and explains the conclusion that *pof1* mutants are defective in cell growth as opposed to cell cycle regulation.

3.1 Production of Pof1 temperature sensitive mutants

Temperature sensitive *pof1*$^+$ alleles were created using mutagenic PCR as illustrated in Figure 3.1. The first step was to create a suitable PCR template. This had to contain the *pof1*$^+$ gene tagged with some marker gene so that strains containing the mutated gene could be selected. For this purpose we utilised the Pof1-GFP strain described in the previous chapter, since this contained *pof1*$^+$ tagged with the kanamycin gene. Genomic DNA was extracted from Pof1-GFP cells and this was used as a template for PCR. The entire 4.5kb *pof1*$^+$*-GFP-kan*^{R}
cassette was amplified firstly using standard PCR, then this product used for a second round of PCR, this time error-prone. PCR can be made error-prone, and thus mutagenic, by altering conditions such as nucleotide or magnesium ion concentration (Cadwell, R.C. and Joyce, G.F., 1992). The mutagenically amplified *pof1*$^+$*-GFP-kan*^{R} fragment was then transformed into wild type cells and integrated into the *pof1*$^+$ locus by homologous recombination. Transformed cells were first selected on G418 containing plates at 26°C, then these colonies replicated to rich media plates and plates incubated immediately at 36°C. These plates contained the dye Phloxine B (5mg/ml), a dye which can be excluded from living cells but stains dead cells, which thus appear red on plates. Colonies which could grow at 26°C but were red at 36°C were selected. This process resulted in the isolation of two ts *pof1* alleles, *pof1-6* and *pof1-12*.

Mutant temperature sensitivity is rescued in a *pof1*$^+$-dependent manner

In order to confirm that the ts phenotype was due to a lack of *pof1*$^+$ function a plasmid dependency test was carried out. As described in the previous chapter, this involves the transformation of *pof1* ts cells with a *pof1*$^+$ plasmid. A plasmid carrying the *leu1*$^+$ marker was used this time, since the *pof1*$^+$ ts cells carried only a *leu1* marker mutation. After transformation with the

plasmid, transformants were selected on plates lacking leucine and then grown in rich liquid media overnight, which contains leucine, giving them the opportunity to lose the plasmid. Approximately 500 cells were then plated onto rich media plates and allowed to form colonies. The resulting colonies were replica-plated to rich media plates at restrictive temperature (36°C) and minimal media plates lacking leucine at non-restrictive temperature (26°C). Temperature sensitivity and ability to grow minus leucine were observed. All cells which were temperature sensitive (approximately 20%) could not grow minus leucine (suggesting that they had lost the plasmid). All cells which were not temperature sensitive could grow minus leucine. Thus rescue of ts phenotype correlated to the presence of the *pof1*$^+$ plasmid. The same experiment carried out with an empty vector confirmed this effect was *pof1*$^+$ specific. This confirmed that a lack of *pof1*$^+$ function caused the ts phenotype of isolated strains.

3.2 Sequencing the pof1 ts alleles

Nucleotide sequencing of the *pof1* gene in these ts mutants showed that both contained two mutations (Figure 3.2). The *pof1-6* allele contained two point mutations in the F-box motif region, F109S and S118P respectively (Figure 3.2-upper). These mutations result in the replacement of a non-polar hydrophobic residue with a polar hydrophilic residue (F-S) and the replacement of a hydrophilic residue with a hydrophobic residue (S-P). These would be predicted to interfere with Pof1 interactions with Skp1. The *pof1-12* allele contained two mutations in the C-terminal region following the F-box motif, K246E and S566G (Figure 3.2-lower). These mutations result in the replacement of a positively-charged residue with a negatively-charged residue (K-E). Both Ser and Gly are hydrophilic. These mutations are situated

in either side of the WD40 repeat domain thus would be predicted to interfere with Pof1-substrate binding.

3.3 Investigating the interactions between Pof1 and Skp1 in temperature sensitive mutants

The location of the mutations in the *pof1-6* allele suggested that binding of Pof1 to Skp1 and the core SCF complex may be abrogated in this mutant. This idea was investigated using an immunoprecipitation experiment. Since a *pof1*-GFP cassette had been used for the construction of the ts mutants the mutant proteins were already tagged with a GFP epitope. Extracts were prepared from *pof1-6*, *pof1-12* and the *pof1*$^{+}$-GFP strain as a control. All strains were grown at restrictive temperature for four hours before preparation of extracts. Immunoprecipitation was carried out using anti-Skp1 antibody and the resulting precipitate analysed for Pof1 levels by western blot using anti-GFP antibody (Figure 3.3, left-hand side). Skp1 levels were analysed using anti-Skp1 antibody. Surprisingly Pof1 in both mutants appeared able to bind to Skp1, since in both strains the amounts of Pof1 pulled down by Skp1 was similar to the amount pulled down in the wild type *pof1*-GFP strain. However, total levels of Pof1 were much greater in the mutants than in the wild type strain (Figure 3.3, right-hand side). Thus it was possible that although Pof1 could bind to Skp1 in the mutants this interaction may be less efficient than in wild type cells. Alternatively it could be simply that the amount of Skp1 antibody was limiting, so a similar amount of Pof1 was pulled down in all strains even though there was much more in the mutants. To try and examine this issue further the reciprocal experiment, using anti-GFP antibody to pull down Pof1 and interacting proteins was attempted, but unfortunately attempts to examine Skp1 levels in these immunoprecipitates failed. The reasons for

the increased Pof1 levels in *pof1* mutants will be described further in Chapters 4 and 6.

3.4 Phenotypic analysis of pof1 ts mutants

Having shown that the growth arrest at 36°C was due to a lack of $pof1^+$ function it was next important to observe the phenotype of the cells at this temperature to get some idea of why they couldn't grow. The cells were grown in liquid media at non-restrictive temperature until exponential phase then shifted to the restrictive temperature for eight hours. Samples were taken every two hours and these samples were processed by measuring cell number, observing cells using phase contrast microscopy and measuring the DNA content of cells using flow cytometry (FACS analysis). Figure 3.4 shows the results from this initial analysis. At restrictive temperature the *pof1* mutants appeared to undergo cell division at a much slower rate than at non-restrictive temperature, hence cell number increased by less than two-fold after eight hours at 36°C, compared to an approximate five-fold increase in the same time at 26°C (Figure 3.4A). Both alleles appeared to arrest growth with an identical morphology as small cells. This phenotype appeared between two and four hours after shift to 36°C. A representative example of this in the *pof1-6* strain is shown in Figure 3.4B. FACS analysis revealed that the majority of theses cells contained 2C DNA content, however after six and eight hours at restrictive temperature a small 1C DNA peak was observed. Again the FACS data was identical in both ts alleles so only *pof1-6* data is shown in Figure 3.4C. Although small, this peak is likely to be significant, since wild type exponentially growing cells never show such DNA content since they are always large enough to copy their DNA whilst undergoing cytokinesis (see Chapter 1). It suggests that a small percentage of *pof1* ts

cells are in G1-phase of the cell cycle after incubation at restrictive temperature.

3.5 *pof1* ts mutants retain constant viability at restrictive temperature

Cell number did not increase when *pof1* ts cells were incubated at the restrictive temperature, suggesting that the cells had lost the ability to divide. This could be because the cells had undergone some lethal event at 36°C or they may have simply exited the cell cycle, in a manner similar to nutritionally starved cells. If this were the case the cells should be able to re-enter the cell cycle and start to divide if transferred back to non-restrictive temperature. To test this the viability of *pof1-6* cells after incubation at 36°C was measured. As shown in Figure 3.5, although *pof1-6* cells had slightly reduced viability compared to wild type even at 26°C, incubation at 36°C had no effect on their viability. Thus the small *pof1-6* cells seen at restrictive temperature were not dead, as they were able to form viable colonies at 26°C.

3.6 *pof1* ts mutants arrest with a reduced cell size

The extent of cell size reduction in *pof1* ts mutants at restrictive temperature was quantified by measuring cell length and width. Since phenotypes were identical all analysis of phenotype was now carried out using the *pof1-6* allele alone. Cells were again grown at non-restrictive temperature then shifted to restrictive temperature and samples taken every two hours. Cells were stained with calcofluor which stains the cell wall material, to enable easier and more precise measurement of cell length. As shown in Figure 3.6 after eight hours cell length was significantly reduced, with cells at 26°C having an

average length of 13.8μm whilst at 36°C this had dropped to 8.4μm. Cell width however remained the same, at 4.4μm (Table 4). Thus overall size of *pof1* mutants was significantly reduced after 8 hours at the restrictive temperature.

3.7 Is the reduced cell size of *pof1* mutants due to a '*wee1*-like' cell cycle defect?

The short cell phenotype of *pof1* mutants was reminiscent of *wee* mutants, in which there is premature activation of the Cdc2 kinase (Nurse, P., 1990), so mitosis occurs before an appropriate cell size is reached. This results in small cells with a G1 DNA content. If *pof1* mutants were somehow inactivating Wee1 kinase activity they would accelerate through mitosis and also arrest as small cells. To investigate this idea further we made double mutants between *pof1-6* and the key regulators of Cdc2 kinase: *wee1, mik1* and *cdc25*. All three double mutants were still ts, suggesting that the *pof1-6* temperature sensitivity was not due to lack of phosphorylation of Cdc2, since it was not rescued by Cdc25 phosphatase inactivation. The length of these mutants at restrictive temperature was next measured. Cells were grown to exponential phase then shifted up to restrictive temperature and length and width of cells measured after 6 hours (Figure 3.7). The average length of *pof1-6* cells was shorter than wild type but longer than *wee1-50* cells (Figure 3.7A) The combination of a *wee1-50* and *pof1-6* mutation also produced small cells, the average length of which was actually slightly shorter than *wee1-50* alone. Finally the double *cdc25-22, pof1-6* mutant appeared to arrest at a cell length somewhere inbetween that of *cdc25-22* and wild type cells, thus *pof1-6* appeared to slightly reduce the length of a *cdc25-22* mutant.

There is no change in Cdc2 phosphorylation levels in *pof1-6* mutants

The fact that a *cdc25-22 pof1-6* double mutant was still temperature sensitive suggested that the ts phenotype of *pof1-6* was not due to reduced Cdc2 phosphorylation. To confirm that Cdc2 Tyr15 phosphorylation was not reduced in a *pof1-6* strain the level of Tyr15 phosphorylation was examined during 4 hours at the restrictive temperature. Although Cdc2 phosphorylation changes during the course of the cell cycle a '*wee*' like mutant should show an obvious reduction in Cdc2 phosphorylation over time even in an asynchronous culture. However *pof1-6* showed no significant alteration in Cdc2 Tyr15 phosphorylation state over the course of four hours at 36°C (Figure 3.8).

3.8 *pof1-6* cells cannot recover from a nitrogen starvation- induced cell cycle arrest

S. pombe cells deprived of nitrogen will enter stationary phase as small cells with a 1N DNA content. The cells remain viable and will re-enter the cell cycle again when exposed to nutrients. The small cell size of *pof1-6* mutants and the fact that they remain viable if shifted back to a non-restrictive temperature is similar to these nutrient-deprived cells. If *pof1-6* cells at 36°C were acting like nutrient-deprived cells they should be unable to re-enter the cell cycle at this temperature after a nitrogen starvation induced arrest. This was tested by arresting the cells in media lacking nitrogen at 26°C, then filtering back into rich media but at 36°C, as summarised in Figure 3.9A. After returning to rich media cell samples were taken every hour for eight hours and cell number, morphology, size and DNA content analysed. The *pof1-6* cells grown at 26°C after return to nitrogen-containing media were able to resume cell division, thus cell number increased (Figure 3.9B). However those grown at 36°C failed to significantly increase cell number, suggesting they were unable to re-

enter the cell cycle. An analysis of the DNA content of cells revealed that although the *pof1-6* cells were starting to increase DNA content after return to rich media, this occurred at a far slower rate than wild type cells (Figure 3.9C) and corresponded to a lack of increase in cell size (Figure 3.9 D and E). These data indicate that *pof1-6* cells have inefficient cell growth and cell cycle progression following nitrogen starvation-induced cell cycle arrest.

3.9 *pof1-6* cells cannot grow at restrictive temperature following G2-phase synchronisation

To further investigate if the *pof1* growth defect occurred during a specific phase of the cell cycle a *pof1-6* culture was synchronised using centrifugal elutriation. A newly formed *S. pombe* cell will be large enough to undergo DNA replication before it has actually separated from its sister cell by cytokinesis, thus cells which have just separated by cytokinesis, therefore the smallest cells in a culture, will be in G2-phase. This property can be used to synchronise cultures, since if the smallest fraction of cells is removed from a culture they will all be in early G2-phase. This technique was used on an exponentially growing *pof1-6* culture, the isolated G2 fraction then split into two and cell growth at 26°C and 36°C observed. Cell number failed to increase significantly at 36°C (Figure 3.10A) although analysis of the number of septating cells in each culture revealed that pof1-6 cells at 36°C did undergo a synchronised wave of septation, albeit at a reduced number compared to 26°C cultures (Figure 3.10B). The fact that cell number did not appear to increase despite septation occurring probably reflects the fact that *pof1-6* cells did not undergo significant growth following division, as indicated by FACS analysis (Figure 3.10C) thus would not be detected by the cell

counter. Prior to division there was some slight increase in size of cells at restrictive temperature, although cells did divide at a reduced size compared to the 26°C sample. Finally measurement of total RNA and protein content of the cultures revealed that over the 4 hour time course both accumulated at a much slower rate at 36°C, indicating that cell growth was defective (Figure 3.10D and E).

Summary

This chapter has described the isolation of two temperature sensitive alleles of the *pof1* gene. These alleles caused an arrest in growth at restrictive temperature, with cells arresting at a reduced cell size, approximately 60%, compared to their average size at non-restrictive temperatures. These alleles were shown to contain mutations in either the F-box motif region or the predicted substrate binding region of Pof1, however both appear able to interact with Skp1.

In an attempt to assess why the *pof1* mutants arrested with a small size the involvement of cell cycle regulators in the phenotype was analysed using genetics. A *cdc25* ts mutation was not able to rescue the growth of the *pof1* mutant at restrictive temperature. Cdc2 Tyr15 phosphorylation state appeared to undergo no obvious change in these conditions.

To attempt to assess at which point in the cell cycle the *pof1* growth arrest was occurring, experiments where the growth of *pof1* ts mutants at restrictive temperature following cell cycle synchronisation were carried out. Nitrogen starvation and elutriation techniques were used to synchronise cells in G1 and G2-phase respectively. In both cases *pof1* mutants were unable to

significantly increase cell size, although a percentage were able to go through some stages of the cell cycle, despite their reduced size.

Discussion

The screen for temperature sensitive *pof1* mutants resulted in the isolation of two ts alleles. The first question which arose from the study of strains carrying these alleles was why they were defective in Pof1 function. The localisation of the mutations in these two alleles is different, with *pof1-6* containing mutations in the F-box motif region, while *pof1-12* contains two mutations in the C-terminal region. The *pof1-6* mutations would be predicted to restrict interactions between Pof1 and the core SCF components whilst *pof1-12* mutations would be predicted to interfere with substrate binding. However the immunoprecipitation experiment suggests this may not be the case. Both mutant Pof1 proteins appeared able to interact with Skp1 with the same efficiency as wild type. It is possible that these interactions are slightly reduced relative to the wild type situation, since there was much more Pof1 protein expressed in the mutant strains, yet the amount precipitated with Skp1 was only equal to that which precipitated in the wild type strain. However this is equally likely to be caused by Skp1 antibody being the limiting factor for Pof1 pull-down. If the Skp1 antibody was saturated with bound Pof1 in the wild type condition, then you could not expect to pull down increased amounts of Pof1 in the mutant strains even though there was more available. The fact that Pof1 in both mutant alleles interacted with Skp1 at a similar efficiency also contradicts the idea that the mutations in *pof1-6* would specifically restrict interactions with the core SCF complex, since if this were correct much less Pof1 should be pulled down by Skp1 in the *pof1-6* background relative to the *pof1-12* background. Thus it is currently unclear why the *pof1* ts alleles are defective but it must be assumed that this is a

defect in substrate interaction or an inability to correctly position the substrate for ubiquitylation rather than a failure to interact with the SCF itself.

The second question to arise from the data in this chapter concerns the cause of the small cell phenotype when the *pof1* mutant arrests growth at 36°C. As discussed in previous chapters small cell size can be caused by defects in cell cycle regulation or actual cell growth. In fission yeast several mutations in cell cycle regulators have been isolated which lead to small cell size, the most extensively analysed of these being *wee1*, a tyrosine kinase which negatively regulates cell cycle progression in G2-phase by phosphorylating Cdc2. Mutations in this gene lead to progression through mitosis before an adequate size is reached, with these small cells eventually arresting in G1-phase through a second size checkpoint. The small size of these cells coupled with the appearance of a very small fraction of cells in G1 initially suggested that *pof1* mutants might be exhibiting a '*wee1*-like' phenotype. However the data presented in this chapter suggests that the *pof1* phenotype is not caused by a reduction in Cdc2 phosphorylation. Crucially, the *pof1-6* mutation does not rescue a mutation in *cdc25*, the tyrosine phosphatase which normally removes the Tyr15 phosphate from Cdc2, thus activating cell cycle progression. Because Cdc25 and Wee1 are normally antagonists, a *cdc25 wee1* double mutant is viable, although phenotypically *wee* (Fantes, P.A., 1981). This is because the *cdc25* mitotic block caused by hyperphosphorylation of Cdc2 is *wee1* dependent. In contrast a *cdc25 pof1* mutant cannot grow at restrictive temperature, suggesting that *pof1* is not responsible for Cdc2 phosphorylation. Thus Pof1 appears not to be a negative regulator of G2/M phase progression. In addition the size of a *cdc25* mutant is reduced when combined with a *pof1* mutation. Since Pof1 is not a negative regulator of the cell cycle this is likely to be caused if macromolecular growth is inhibited in *pof1-6*.

The data from the synchronisation experiments also suggest some cell growth defects in *pof1-6*. Cell size fails to increase efficiently following a nitrogen starvation induced growth arrest and G2-phase elutriated *pof1-6* cells can increase size in the first 80 minutes following the shift to 36°C, sufficient to go through one round of division, but then show no further size increase. Both of these experiments again point to an inability of *pof1-6* cells to increase their mass rather than hyperactive cell cycle progression. In both experiments a fraction of *pof1-6* cells appear to have uncoupled cell size from cell cycle progression; the cells in the G1 arrest experiment appear to be gradually moving into G2-phase despite not increasing their size and the cells in the elutriation experiment undergo division at a smaller size at restrictive temperature. Although this suggests that it is possible that Wee1 activity is slightly compromised in *pof1* mutants, this is also possibly due to the cell size controls themselves. Cells in starvation conditions have been shown to modulate size controls so that they divide at a reduced size (Fantes, P. and Nurse, P., 1977). It has been suggested that this is caused by a decrease in cell growth leading to a reduction in translation of cell cycle regulators. The decrease in size of *pof1-6* cells at cell cycle transitions is probably due to some modulation of the size checkpoints because of the arrest in cell growth in these mutants and does not necessarily mean that the controls are actually defective.

Thus, in summary, the data presented here suggests Pof1 has a role in cell growth. This could be in detecting growth conditions to switch growth machinery on, or Pof1 could be involved directly in some fundamental growth process itself. The fact that Pof1 is an F-box protein, thus likely to be responsible for the inactivation of a substrate, suggests that a substrate of Pof1 could be a growth inhibitor.

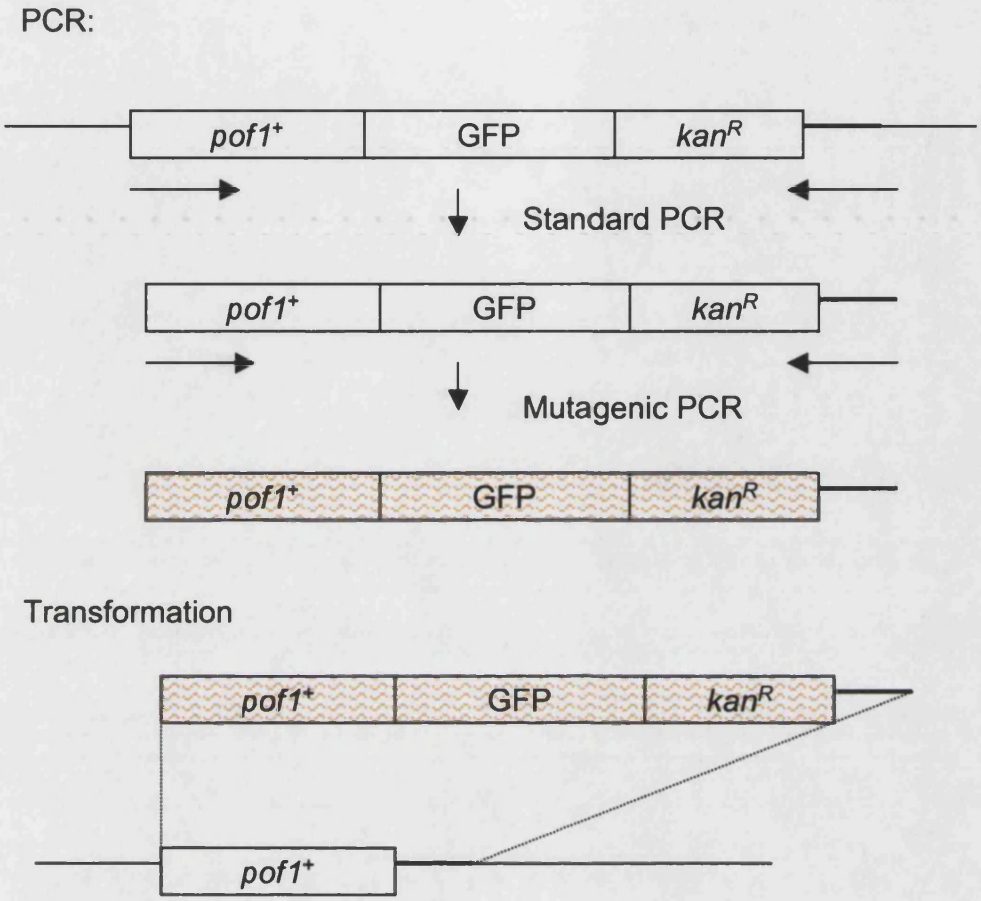


Figure 3.1 The production of *pof1* temperature sensitive mutants.

The 4.5kb *pof1*$^{+}$-*GFP*-*kan*^{R} cassette was amplified using standard PCR, then this fragment used as a template for mutagenic PCR using error prone PCR conditions. The mutagenised fragment (shown in orange) was transformed into wild type cells and integrated by homologous recombination. The thick black line indicates region of sequence homology between fragment and sequence C-terminal to *pof1*$^{+}$

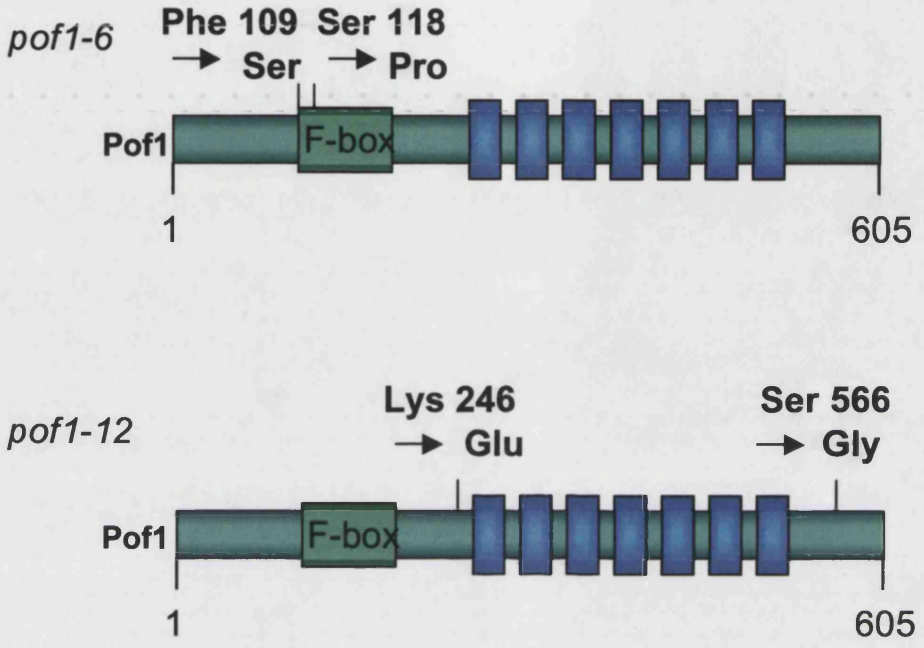


Figure 3.2 Mutation sites in the *pof1* ts mutants.
Schematic diagram shows location of mutations in the two *pof1* ts alleles isolated. Approximate location of the F-box domain and seven WD40 repeats are illustrated with boxes.

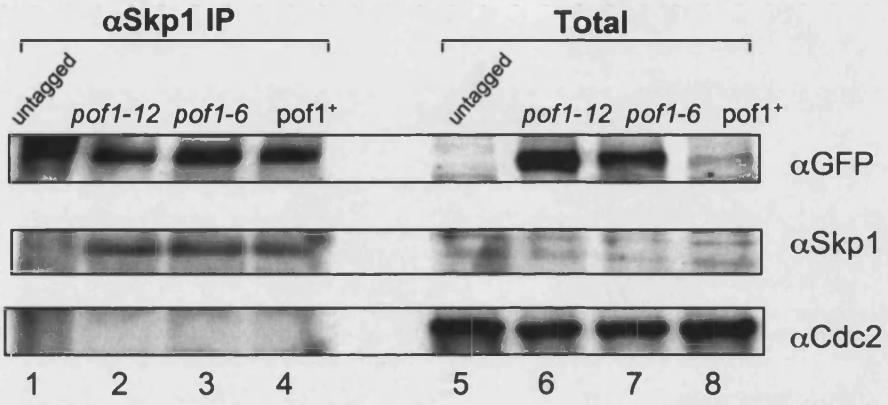


Figure 3.3 Binding between Pof1 and Skp1 in *pof1* ts mutants.

Immunoprecipitation was performed with anti-Skp1 (lanes 1-4) using protein extracts prepared from untagged (lane 1) *pof1-12* (lane2), *pof1-6* (lane 3) and *pof1*$^{+}$-GFP strains (lane 4), which were incubated at 36°C for 4h. Total extracts from the same strains were also run (lanes5-6). Immunoblotting was performed with anti-GFP (upper), anti-Skp1 (middle) or anti-Cdc2 (lower).

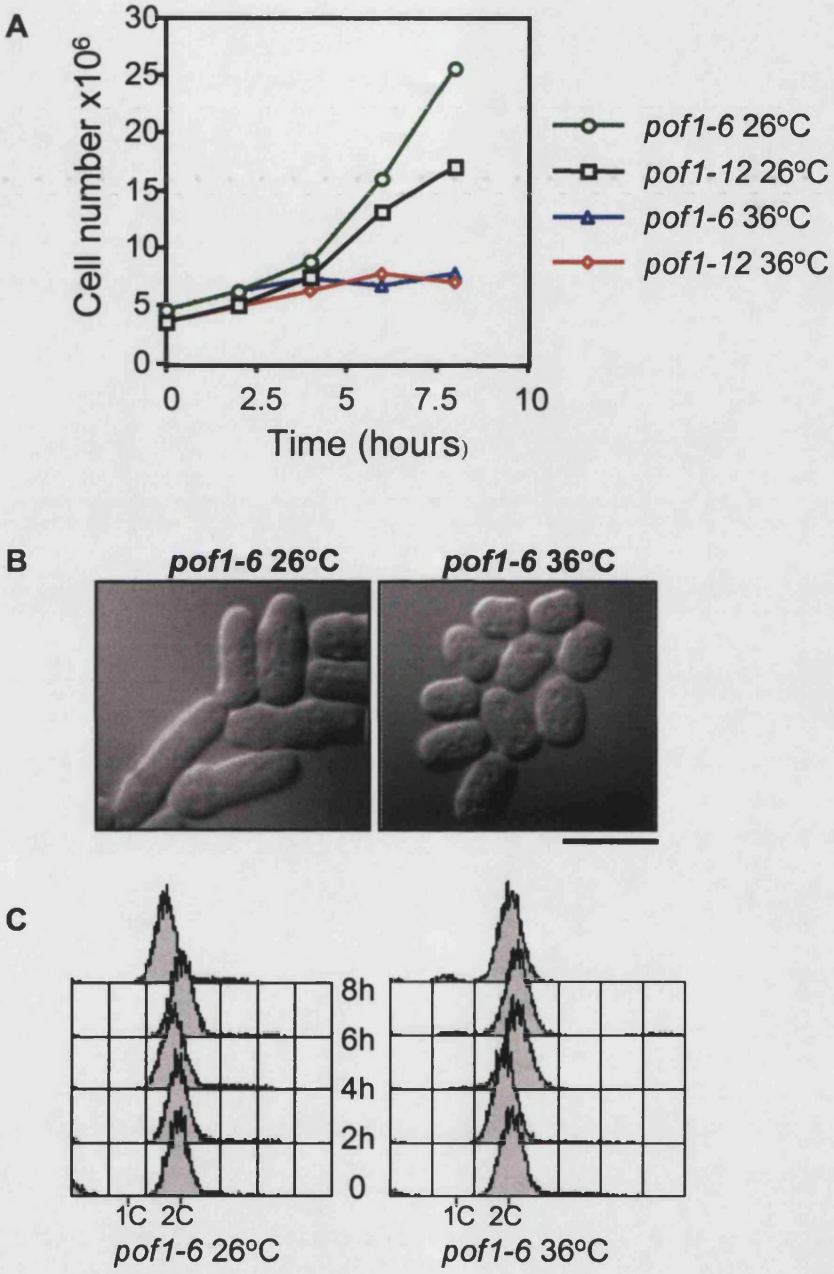


Figure 3.4 The phenotype of *pof1* ts mutants.

pof1 ts strains were grown at 26˚C overnight, cultures split into two and half shifted to 36˚C then incubated for 8h. Samples were taken every 2 hours. **A.** Cell number of *pof1-6* and *pof1-12* cultures **B.** Phase contrast microscopy images of *pof1-6* cells at 6 hours at 26˚C (left) and 36˚C (right) The bar indicates 10 μm. **C.**DNA content of *pof1-6* strain grown at 26˚C (left) and 36˚C (right) . Note that a small G1 (1C) peak appeared at 6 and 8h at 36˚C.

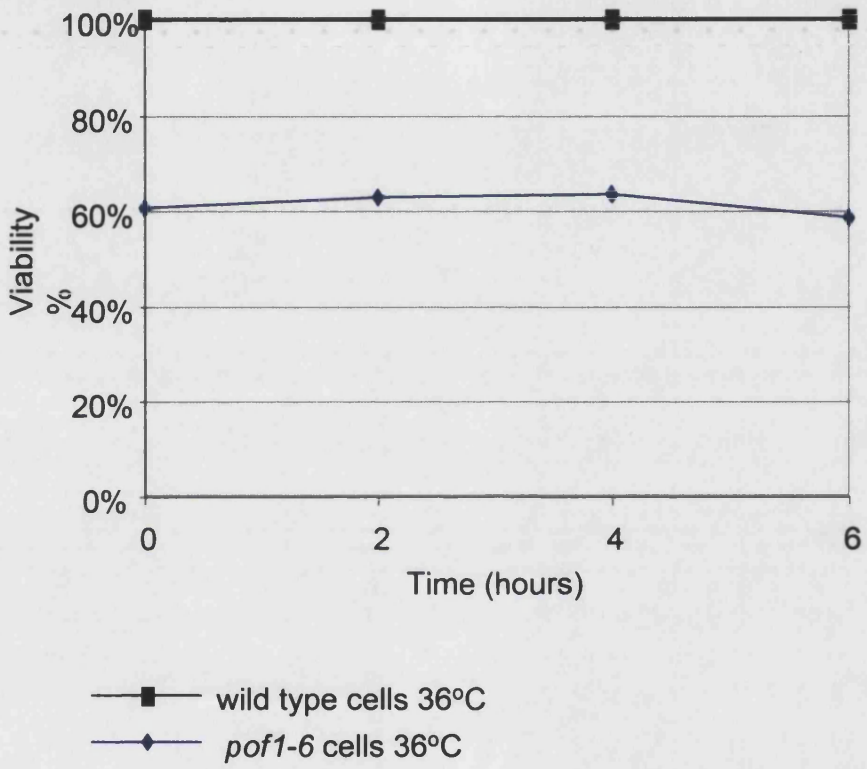
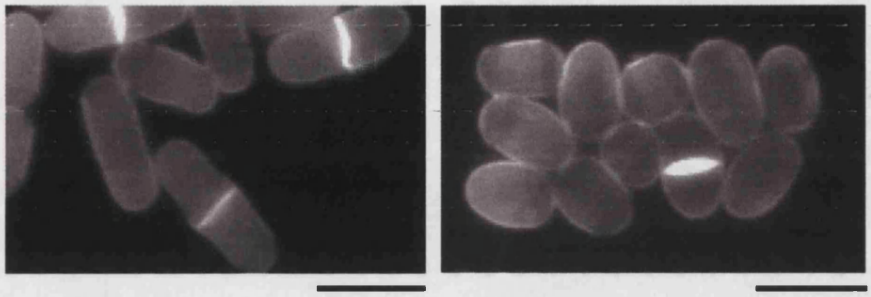


Figure 3.5 The viability of *pof*1 mutants does not decrease at restrictive temperature

pof1-6 cells were grown at 26°C to exponential phase, then shifted to 36°C for six hours. Samples were taken every 2h and 200 cells plated onto rich media plates and incubated at 26°C. Graph shows percentage of viable colonies which grew after 5 days incubation.

A.



B.

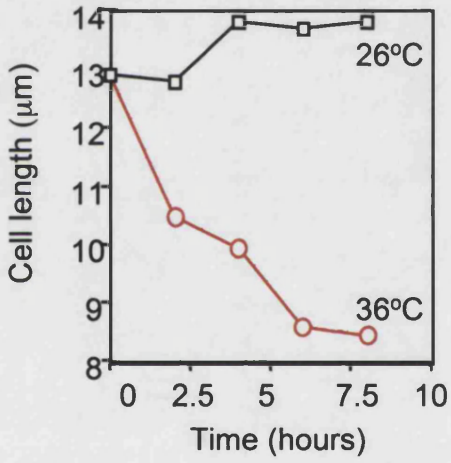


Figure 3.6 Cell size decreases in *pof1* mutants at restrictive temperature

A. *pof1-6* strain was grown at 26˚C overnight, culture split into two and half shifted to 36˚C then incubated for 8h. Samples were taken every 2 hours and stained with calcofluor. Cells from 8h 26˚C (left) and 36˚C (right) are shown. The bar indicates 10 μm **B.** Length of at least 100 cells from each time point was measured.

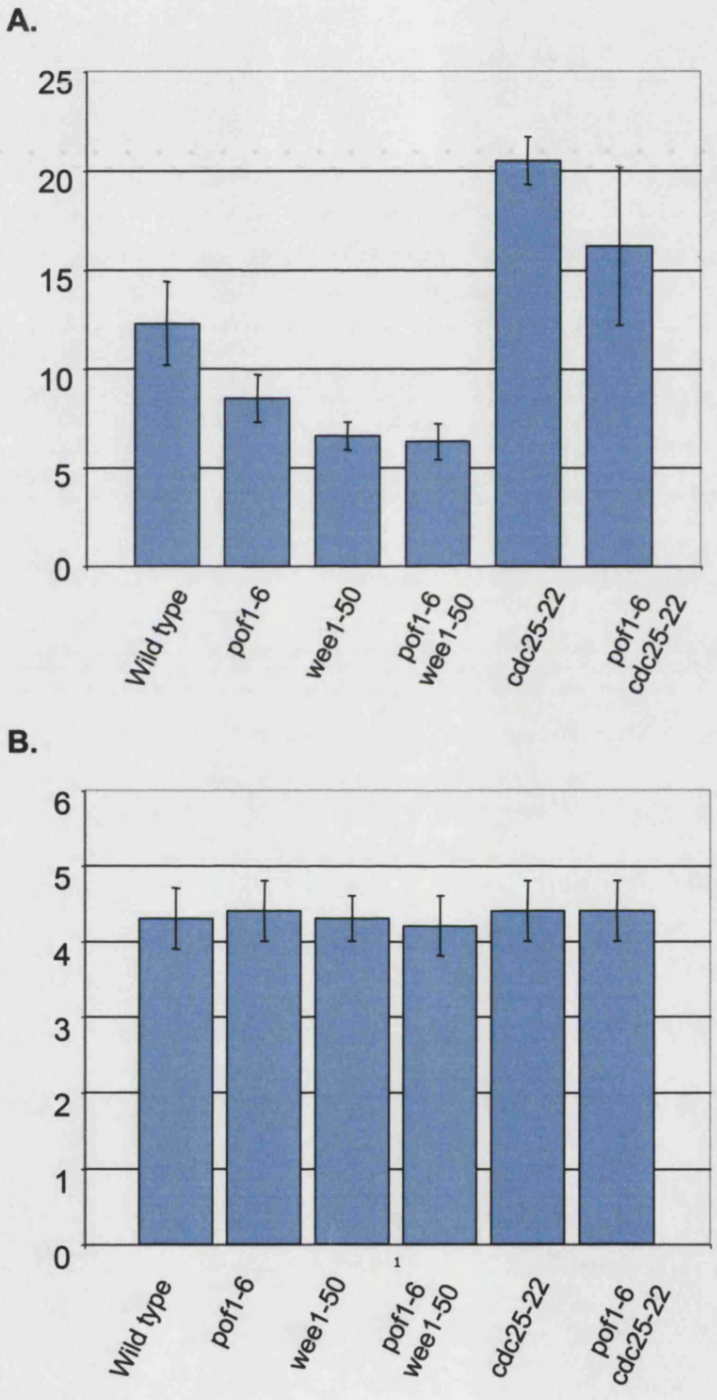


Figure 3.7 Cell size of *pof1* mutants combined with *wee1* or *cdc25* mutation.

Cells were grown overnight at 26°C then shifted to 36°C for eight hours. Length and width of 100cells of each strain was measured.

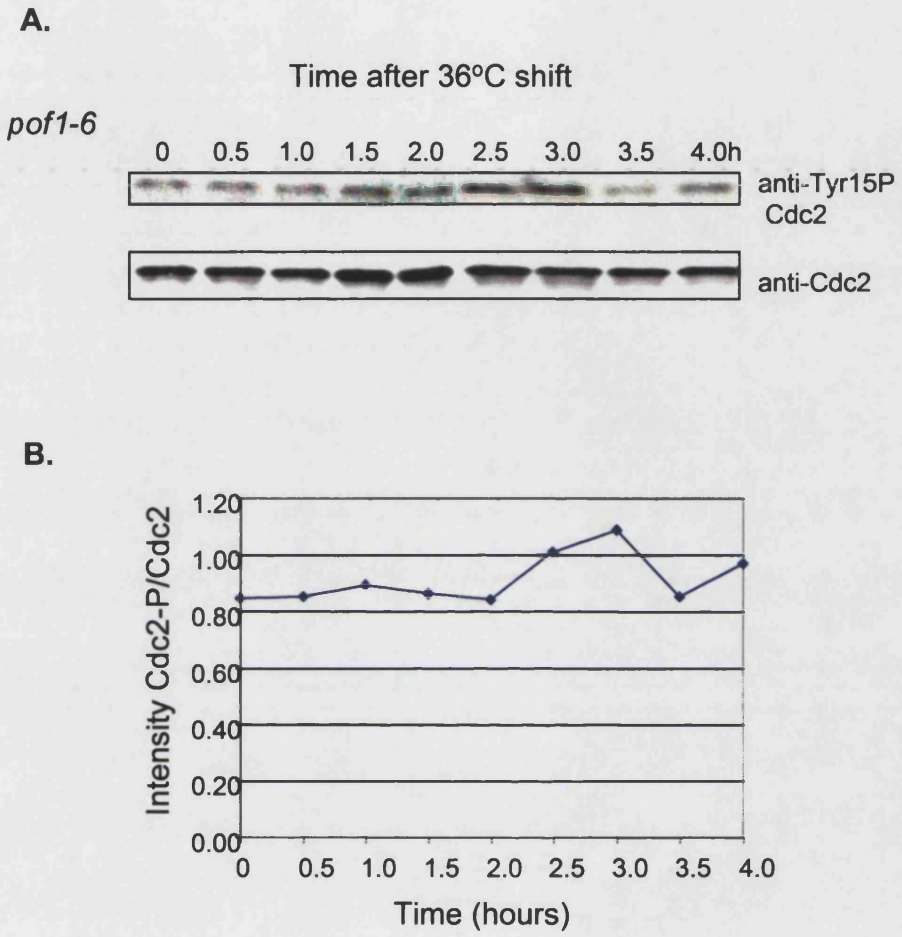


Figure 3.8. Examination of Cdc2 Tyr15 phosphorylation levels in *pof1-6* mutant at restrictive temperature.

A.*pof1-6* strain was grown at 26°C then shifted to 36°C for 4 hours. Samples were taken every 30 minutes, protein extracts made and these extracts examined for levels of total Cdc2 (lower panel) and Cdc2 phosphorylated onTyr15 residue (upper panel). **B.** Quantification of Cdc2 Tyr15-P levels relative to total Cdc2 levels shown in A.

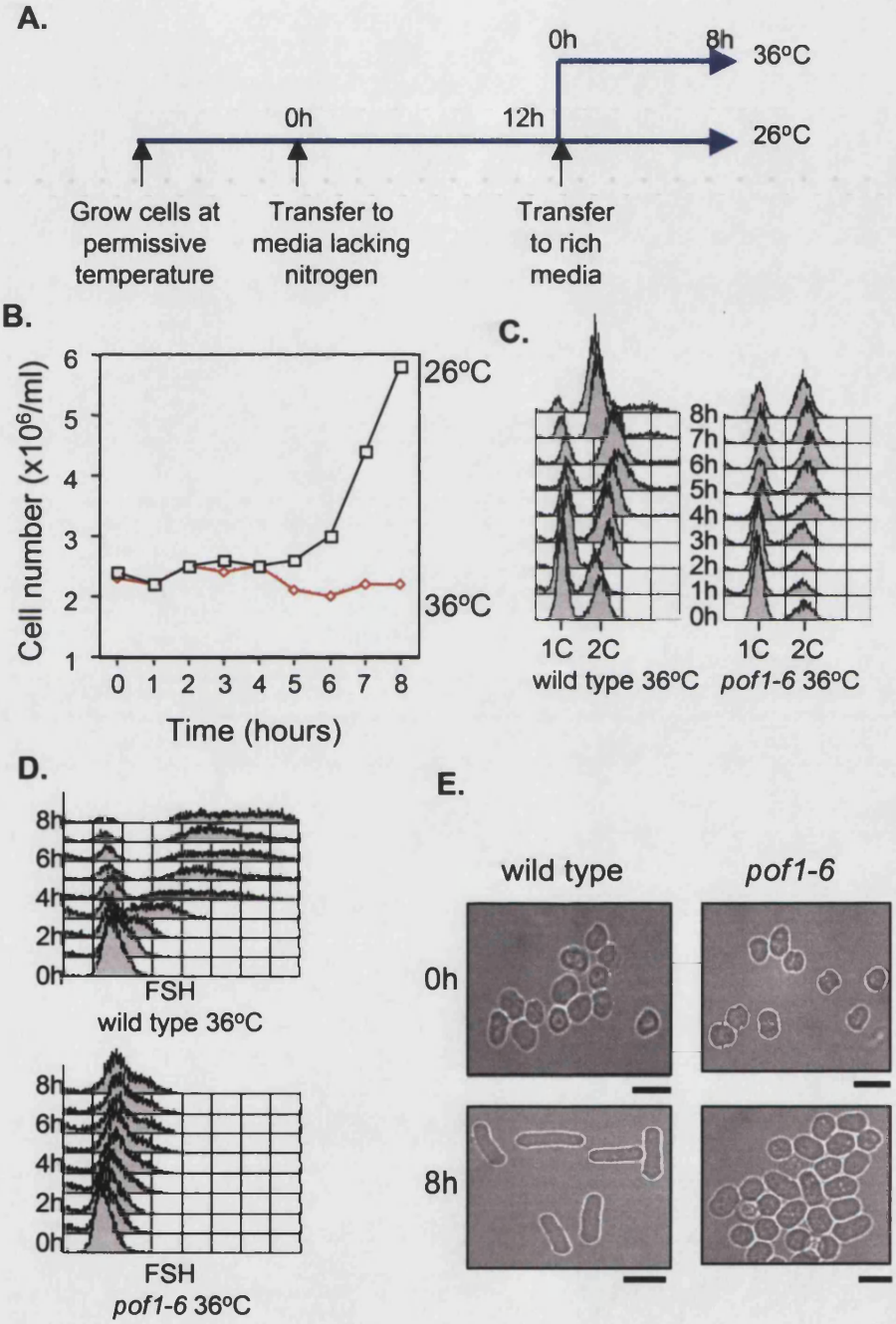


Figure 3.9 *pof1-6* cells cannot efficiently recover from a nitrogen-starvation induced G1 phase arrest.

A. *pof1-6* and wild type cells were grown to exponential phase at 26°C, shifted to low-nitrogen media for 12h to arrest cells, then back into rich media but half of cell cultures were at 36°C **B.** Cell number of *pof1-6* cells grown at 26°C and 36°C following release from nitrogen starvation **C.** DNA content of wild type (left) and *pof1-6* (right) cells at 36°C analysed by FACS **D.** Cell size of wild type (left) and *pof1-6* (right) at 36°C analysed by FACS **E.** Light microscopy images of wild type (left) and *pof1-6* (right) cells at 0h after nitrogen starvation (upper panels) and 8h after release at 36°C

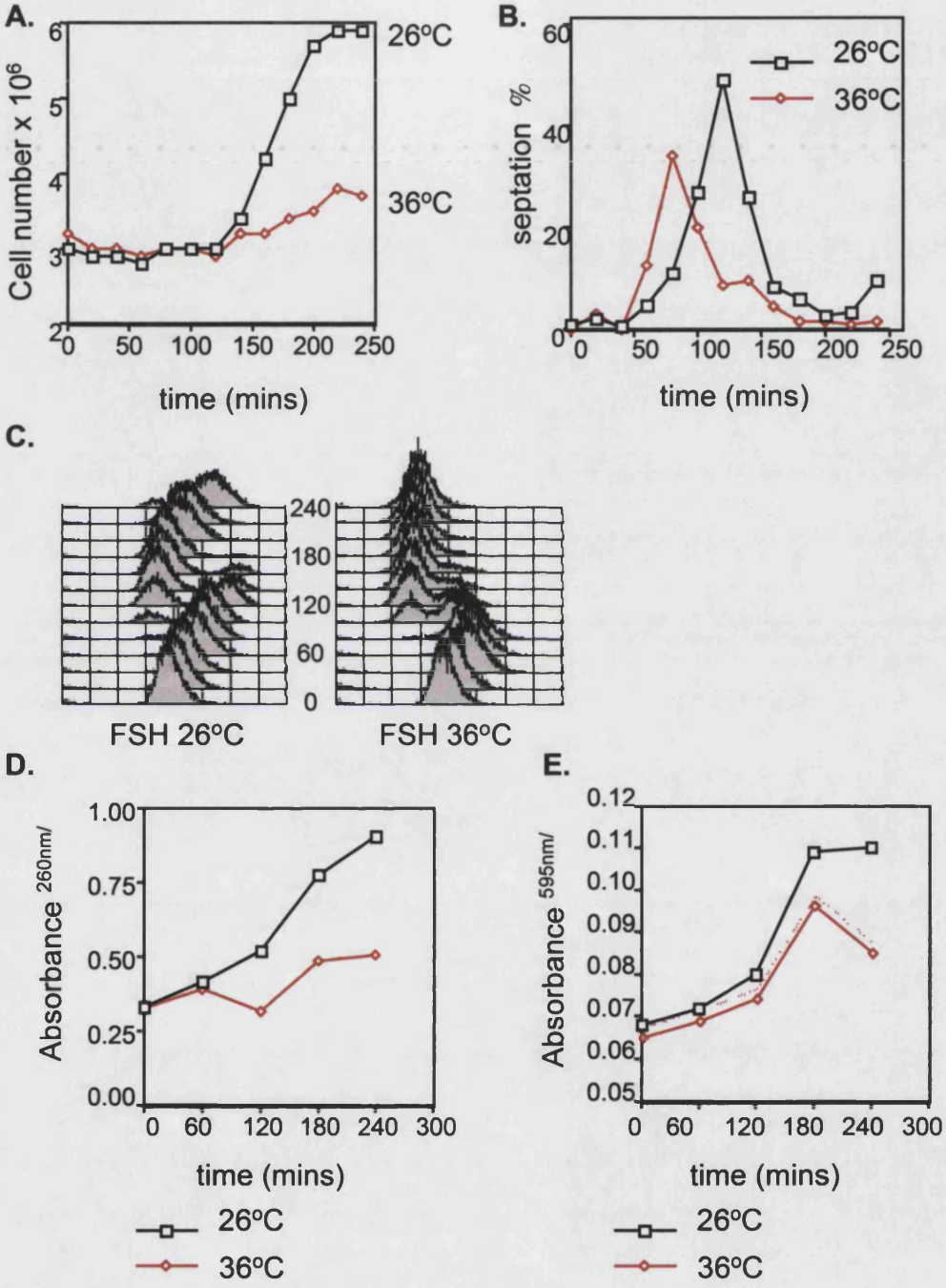


Figure 3.10 *pof1-6* cells cannot grow at restrictive temperature following a G2 phase synchronisation.

pof1-6 cells in G2 phase were isolated from an exponentially growing culture, and grown at 26°C or 36°C. Samples were taken every 20 minutes and measured for **A.** Cell number **B.** Percentage of cells septating **C.** Cell size by FACS **D.** Total RNA content **E.** Total protein content.

Table 4. Cell size of the *pof1-6* mutant at restrictive and non-restrictive temperature

Timepoint	cell length μm	cell width μm
8h 26°C	13.8 ± 1.8	4.4 ± 0.4
8h 36°C	8.4 ± 1.2	4.4 ± 0.3

Exponentially growing strains at 26°C were shifted to 36°C and incubated for 8h. At least 100 cells were measured for each sample

Chapter 4

Isolation of a suppressor of *pof1* temperature sensitive mutants

Introduction

The previous chapter discussed the isolation of *pof1* ts mutants and the phenotype of these mutants at restrictive temperature. Since Pof1 is an F-box protein it was highly probable that the growth arrest seen at this temperature was caused by some substrate accumulating in the mutants. To try and identify this substrate an extragenic suppressor screen was carried out, on the basis that inactivation of this substrate would rescue the growth of the mutants at restrictive temperature. This chapter describes the suppressor screen, the isolation of a mutation which could rescue the *pof1* mutant temperature sensitivity and the subsequent cloning of this suppressor gene.

4.1 The screen for a suppressor of the *pof1* temperature sensitive mutants

Since Pof1 is an F-box protein and had been shown to interact with core SCF components it was logical to assume that in wild type cells it was responsible for the inactivation of some substrate and that the inability of *pof1* ts mutants to grow at restrictive temperature was due to inappropriate activation of this substrate. Pof1 is homologous to Met30, known to inactivate its substrate, Met4 through proteolytic and non-proteolytic mechanisms. To try and identify the substrate of Pof1 I had carried out extensive searches of *S. pombe* gene and protein databases using Met4, to attempt to find a sequence homologue of Met4. However all attempts had resulted in no obvious Met4 homologue being found. Thus a genetic approach was used to try and identify the substrate of Pof1.

The *pof1-6* strain was grown to exponential phase in liquid culture. The cells were then plated onto 20 rich media plates, at approximately 1x 10^7 cells per plate. These plates were incubated at the restrictive temperature, 36°C, for several days. Most of the cells on the plates should be unable to grow and form colonies since they contained the *pof1-6* mutation, however any colonies which did grow must have either mutated the *pof1* gene itself so it could function again as wild type or have mutated some other gene which allowed them to grow, the most likely candidate being the substrate itself. This idea is illustrated in Figure 1.4. After 10 days at 36°C a total of 23 colonies had grown on the plates.

Identifying intragenic or extragenic suppressors

The first thing to establish with these strains was whether the mutation which allowed them to grow at 36°C was a mutation within the *pof1* ORF which had

somehow allowed it to produce functional Pof1 (intragenic mutation) or some mutation in a separate, and therefore substrate candidate, gene (extragenic mutation). If the mutation was within the *pof1* ORF and the strain was mated with wild type cells, none of the offspring from this mating should contain the original *pof1-6* mutation, since it would be impossible for cells to separate this from the intragenic mutation, thus no cells should be temperature sensitive. If, however, the suppressor mutation was extragenic to *pof1* the suppressor mutation and the *pof1-6* allele should be inherited in the offspring in a Mendelian fashion, thus the offspring should contain temperature sensitive, kanamycin resistant cells, as illustrated in Figure 4.2. Thus the suppressor strains were mated with wild type strains, the resulting spores plated onto non-selective rich media and allowed to grow into colonies. These colonies were checked for temperature sensitivity and kanamycin resistance by replica plating as described previously. Using this approach the 23 suppressor strains were separated into two groups, 9 strains which contained some intragenic *pof1* mutation and 14 strains which contained an extragenic suppressor mutation.

Testing whether all extragenic suppressors correspond to the same locus

The next step was to check whether all of the 14 extragenic *pof1* suppressor mutations corresponded to the same locus. One of these suppressor strains was selected and mated individually with the other 13 strains. Fission yeast strains must be of different mating types to be able to mate. All suppressors were isolated in the same strain background so were the same mating type, however since all of the suppressors had been previously crossed with wild type strains it was possible to isolate one of the extracellular suppressor strains with the opposite mating type to the other 13. This experiment was based on a similar genetic approach to the previous one, in this case if the two strains contained suppressor mutations in the same locus all offspring

would contain the mutation and no temperature sensitive offspring would be produced, however if the suppressor mutations were in different loci some of the offspring would inherit neither and thus be temperature sensitive. When one of the suppressor mutants was crossed individually with the other 13 none of the crosses resulted in temperature sensitive progeny. This indicated that all 14 suppressor strains contained a mutation in the same locus. Thus the screen had resulted in the isolation of a single suppressor locus of *pof1-6*. This locus is from this point on referred to as *sup9*.

Isolation of the suppressor mutation

It was next necessary to separate the *sup9* locus from the *pof1-6* locus. If a strain containing just a *sup9* mutation could be isolated it might be possible to clone the *sup9* gene. This was carried out by again crossing one of the *pof1-6 sup9* strains with wild type, but then carrying out tetrad dissection of the resulting sporulating diploids. Tetrad dissection allows you to see each of the four daughter cells from an individual diploid. Thus it was possible to find a tetrad where complete segregation between the *sup9* and *pof1-6* alleles had occurred (known as non-parental ditype), because this tetrad would contain two temperature sensitive kanamycin resistant colonies, representing *pof1-6* strains, and two non-temperature sensitive, kanamycin sensitive colonies which had to contain the *sup9* mutation. Thus by genetic analysis a strain containing a mutation able to suppress the ts phenotype of *pof1-6* was isolated.

4.2 Cloning the suppressor of *pof1*

Now that a strain had been isolated containing a mutation which could suppress the *pof1* ts phenotype it was important to identify which gene this mutation was in. In order to clone the gene it was necessary to find some

condition where the mutation inhibited normal growth. The *sup9* strain appeared to grow normally at all temperatures. A study in fission yeast to identify genes expressed in response to various stress conditions had identified *pof1*$^+$ as a gene specifically expressed in response to cadmium stress (Chen, D. et al., 2003). If the suppressor was a substrate of Pof1 it was possible that it was also involved in cadmium stress response. Thus the growth of *sup9* cells in response to cadmium was tested by plating serial dilutions of *sup9*, wild type and *pap1*-deleted cells (Toone, W.M. et al., 1998) as a negative control, onto plates containing cadmium sulphate and incubating at 26°C for several days to compare growth. As shown in Figure 4.3 *sup9* cells were very sensitive to cadmium, growing less efficiently than wild type and the negative control. After testing various cadmium concentrations it was found that 1.0mM was sufficient to prevent growth of *sup9* colonies when lawns of cells were plated from liquid culture. The *sup9* strain was transformed with a plasmid-based genomic library and 1 x 10^7 cells per plate spread onto 30 plates containing 1.0mM cadmium sulphate. Two independent transformants were able to grow on these plates. Plasmid dependency tests were carried out as described previously and these confirmed that the ability of the transformants to grow in the presence of cadmium was plasmid dependent. The plasmids were then extracted and regions at each end of their inserts sequenced. Comparison of these sequences against the *S. pombe* genomic sequence, using the sequence data from the Sanger Centre, revealed both contained identical inserts, containing three complete ORFs: *ydc2*$^+$ (SPAC25G10.02) a Holliday junction resolvase component, *rec10*$^+$ (SPAC25G10.04c) involved in meiotic recombination and *zip1*$^+$ (SPAC25G10.03), a bZIP transcription factor. Zip1 was an obvious candidate for the substrate of Pof1, since the *S. cerevisiae* homologue of Pof1, Met30, was already known to regulate a bZIP transcription factor, Met4. *zip1*$^+$ was originally isolated in a screen for multicopy suppressors of the calcium sensitivity of fission yeast mutants

lacking the activity of another bZIP transcription factor, Atf1 (Ohmiya, R. et al., 1999). However the function of Zip1 was unknown. The *zip1* ORF was sequenced in the *sup9* suppressor strain and found to contain a GGA to TGA nonsense mutation corresponding to amino acid residue glycine 189. This resulted in the insertion of a STOP codon in the middle of the *zip1* ORF prior to the C-terminal bZIP region (Figure 4.3 B). Thus this nonsense *zip1* allele was likely to be the suppressor of *pof1-6*.

4.3 Analysis of the genetic interactions between *zip1* and *pof1*

The fact that the nonsense *zip1* allele was able to suppress the *pof1-6* phenotype suggested that increased Zip1 activity may be the cause of the *pof1-6* growth arrest. To investigate this idea I first checked whether *zip1* could rescue the other *pof1* ts allele, *pof1-12* and the $pof1^+$ deletion. The *zip1* strain was crossed with the *pof1-12* strain. The resulting spores were plated onto non-selective, rich-media plates. After five days at 26°C colonies had grown. These were replica plated onto fresh plates; one replica on rich media was analysed for temperature sensitivity at 36°C and another on G418 plates was analysed for kanamycin resistance (the marker for the *pof1-12* allele) by random spore analysis. Out of a total of 564 colonies, 221 were kanamycin resistant and 134 were not ts (ts^+). This suggested that the *zip1* allele could rescue the temperature sensitivity of *pof1-12*. The *zip1* strain was next crossed with a $pof1^+$-deleted strain. Since $pof1^+$ is essential a strain carrying a $pof1^+$ plasmid was used. This was the $pof1^+$::$ura4^+$ strain carrying a $pof1^+$ plasmid with a *LEU2* marker described in Chapter1. Again random spore analysis was used to look for $ura4^+$ cells that had lost the *LEU2* plasmid. Out of 331 colonies which grew on non-selective plates, there were 43 ura^+ leu^- colonies. To confirm that these colonies really contained a $pof1^+$ deletion 5 were selected and analysed by colony PCR, using one primer just upstream

of the *pof1*$^+$ ORF and the opposing primer specific for either the *ura4*$^+$ ORF or the *pof1*$^+$ ORF. All five colonies only produced bands with the *ura4*$^+$ specific primer, confirming they contained the *pof1*$^+$ deletion. Thus it appeared that *zip1* could rescue the growth of *pof1*$^+$-deleted cells.

Deletion of *zip1*$^+$ rescues growth of *pof1-6* at restrictive temperature

To directly confirm that inactivation of Zip1 suppresses *pof1-6* temperature sensitivity, a complete deletion of the *zip1+* ORF was constructed and crossed with a *pof1-6* strain. The *zip1*$^+$ deletion was constructed using the gene replacement method described in Chapter 1, replacing *zip1*$^+$ with the *ura4*$^+$ ORF (see Appendix A for primer sequences and location). As expected, a haploid strain containing a complete deletion of *zip1*$^+$ was viable. Double mutants were constructed by crossing this *zip1Δ* strain with a *pof1-6* strain containing a *ura4* mutation, selecting kanamycin resistant, ura$^+$ cells by random spore analysis and then confirming *zip1*$^+$ deletion by colony PCR. The *pof1-6 zip1* double mutant could grow at 36°C (data not shown), verifying that loss of Zip1 function rescues temperature sensitivity of *pof1-6*.

4.4 *zip1* overexpression is toxic to *pof1-6* cells at non-restrictive temperature

If Zip1 accumulation or activation was a direct cause for the phenotypic appearance of a *pof1-6* strain at the restrictive temperature, further overexpression of the *zip1*$^+$ gene in this mutant might result in a similar phenotype even at the permissive temperature. To address this, the *zip1*$^+$ gene was subcloned into a multicopy plasmid under the control of a thiamine-repressible mild promoter *(nmt41-zip1*$^+$) (Basi, G. et al., 1993).This plasmid was toxic to only *pof1*-6 cells, but not wild type, at 26°C and arrested cells mimicked the phenotype normally only seen at 36°C, small cell size (Figure

4.4). To investigate the effect of even higher levels of Zip1 activity in the cell, a strain was constructed where the endogenous *zip1*$^+$promoter was replaced with an even stronger thiamine-repressible promoter (*nmt1-zip1*$^+$) in a wild type background (see Appendix A for primer sequences and location). In zero thiamine conditions growth was inhibited even these cells and the cells arrested with a small cell phenotype similar to *pof1* mutants (Figure 4.5). Taken together these data suggest that the small-size phenotype and temperature sensitivity of *pof1* mutants can be explained by an excess or hyperactivation of the *zip1*$^+$ gene product.

4.5 The accumulation of Pof1 in *pof1* ts mutants is *zip1*$^+$-dependent

Previous analysis of Pof1 levels in the *pof1* ts mutants had shown that Pof1 total levels increased in these cells at restrictive temperature (Chapter 3.3, Figure 3.3). The *S. cerevisiae* homologue of Pof1, Met30, is transcribed by its own substrate, Met4, in a feedback mechanism which presumably ensures that the cell always contains sufficient Met30 for any Met4 degradation required (Rouillon, A. et al., 2000). If the relationship between Pof1 and its substrate was similar the increased Pof1 levels seen in the mutant would presumably be due to an accumulation of its substrate and hence an increase in *pof1* transcription. If Zip1 was this substrate the nonsense *zip1* allele should produce non-functional transcription factor and hence the feedback mechanism would no longer occur and Pof1 levels should be reduced in the *pof1-6 zip1* double mutant. Since Pof1 was already tagged with a C-terminal GFP epitope in the *pof1* mutant the levels of Pof1 in both the single and the double mutant could be compared. Both strains along with a *pof1*$^+$-GFP strain were cultured at 26°C and 36°C for four hours and the levels of Pof1 compared by immunoblotting (Figure 4.6). Cdc2 levels were also compared

as a control. At restrictive temperature *pof1-6* cells had clearly increased Pof1 levels compared to wild type cells, but *pof1-6 zip1* double mutants showed similar levels of Pof1 to wild type. This suggests that Pof1 accumulation in *pof1* mutants is Zip1 dependent and supports a model where Zip1 can upregulate *pof1*$^+$ expression in a feedback mechanism.

Summary

This chapter has described a suppressor screen which was carried out using the *pof1-6* mutant to try and identify the gene product which was responsible for the growth arrest of these mutants at restrictive temperature. The screen isolated a single extragenic locus which could suppress the *pof1-6* temperature sensitivity. This suppressor was cloned and found to be a bZIP transcription factor, Zip1. A nonsense *zip1* allele can rescue the growth of *pof1-6* and *pof1-12* ts alleles at restrictive temperature and the growth of cells deleted for *pof1*$^+$. Mild *zip1*$^+$ overexpression is toxic to *pof1-6* cells at non restrictive temperature and more severe *zip1*$^+$ overexpression is toxic to wild type cells. In both cases the cells arrest growth as small cells which look remarkably like *pof1* ts mutants at 36°C. Taken together these data indicate the *pof1* ts growth arrest and small cell phenotype is the result of an increase in Zip1 activity. Furthermore, the increased levels of Pof1 seen in the ts mutants are *zip1*$^+$ dependent, suggesting that Zip1 may regulate *pof1*$^+$ in a transcription feedback mechanism.

Discussion

The isolation of a nonsense *zip1* allele as a suppressor of *pof1-6* ts mutants suggests that too much Zip1 activity is the cause of the temperature

sensitivity and growth arrest of *pof1-6* cells. The fact that *pof1-6* cells are sensitive to a slight increase in Zip1 levels even at non-restrictive temperature further supports this idea. Since Pof1 is an F-box protein, it is likely that Zip1 is a substrate of SCF^{Pof1} and the mutants are defective in the inactivation of this substrate. It is of course possible that Zip1 is not a direct Pof1 substrate, but that Pof1 indirectly represses Zip1 by downregulating the activity of some Zip1 co-activator for example. However the fact that the *S. cerevisiae* Pof1 homologue Met30 has been shown to directly inhibit a transcription factor, Met4, makes Zip1 a likely target for Pof1.

Although both are bZIP transcription factors Met4 and Zip1 are not highly conserved and Met4, at 672 amino acids, has a much larger region N-terminal to its bZIP domain than Zip1, which is only 330 amino acids in length. However, the fact that a *zip1* mutant is cadmium sensitive suggests there is some functional homology between the two transcription factors, since Met4 has been reported to have a role in the transcriptional response to cadmium stress by increasing the transcription of genes necessary for glutathione production (see Chapter 1).

The fact that the *zip1* nonsense allele rescues the growth of cells deleted for $pof1^{+}$ suggests that it is the only substrate Pof1 must degrade for cell viability in normal conditions. It is possible for SCF complexes to have more than one substrate and, in fact, this is commonly seen in F-box proteins with WD40 repeat substrate-binding domains. The *S. cerevisiae* F-box protein Cdc4, for example, regulates at least four known substrates, Cdc6, Gcn4, Far1 and Sic1 (Drury, L.S. et al., 1997; Feldman, R.M. et al., 1997; Henchoz, S. et al., 1997; Meimoun, A. et al., 2000; Sanchez, M. et al., 1999; Verma, R. et al., 1997). However Met30 seems to be essential only because of its role in Met4 regulation, thus *met4Δ* rescues *met30Δ* cells (Patton, E.E. et al., 2000). Pof1 appears to be similar to Met30 in this respect, although it is possible that both

degrade some alternative substrate but that this only becomes essential in specific conditions, such as when the cell is exposed to stress.

The fact that Pof1 accumulation in *pof1-6* mutants is *zip1*$^+$-dependent suggests a feedback mechanism exists between the two gene products. As illustrated in Figure 4.7, this would prevent Zip1 activity ever getting too high, since any increase in active Zip1 would lead to an increase in *pof1*$^+$ transcription and a subsequent downregulation of Zip1. Given that too much Zip1 activity appears to be toxic to cells, as seen in the overexpression experiments described above, this is probably a necessary safety mechanism to prevent Zip1 overactivity. A similar feedback mechanism exists in *S. cerevisiae* between Met30 and Met4. It has been proposed that cells use this to regulate the methionine pathway; when downstream products of this pathway are at high levels in the cell Met30 transcription is increased through Met4, causing a drop in Met4 activity and switching off transcription of the methionine pathway genes (Rouillon, A. et al., 2000). It is likely some kind of similar external modulation of the feedback mechanism in *S. pombe* also exists.

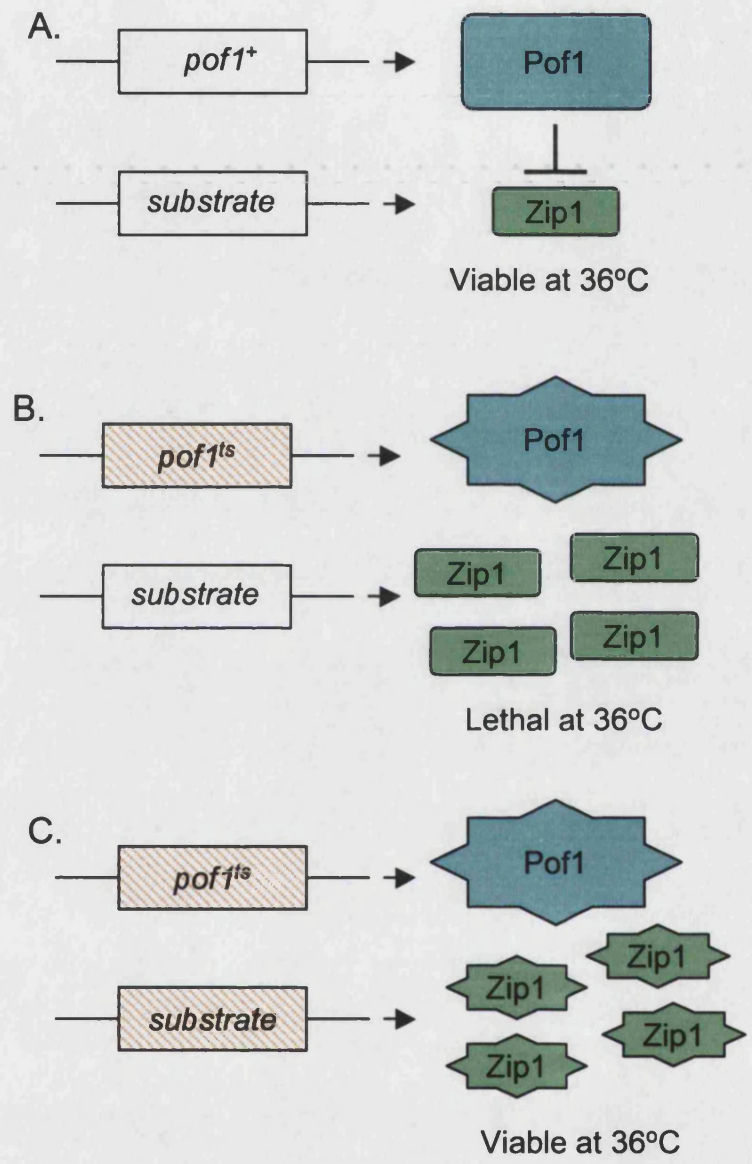


Figure 4.1 Illustration of the theory of the *pof1* suppressor screen.

A In wild type cells Pof1 is responsible for the downregulation of some substrate, presumably through its role in an SCF complex. **B** If this downregulation does not occur, such as when *pof1* is inactivated in a ts mutant, the substrate increases in activity, and this is toxic to the cell **C** If the cell can spontaneously mutate the gene coding for the Pof1 substrate *pof1* ts cells can grow again at restrictive temperature.

Orange shading represents a mutated gene, white boxes are wild type genes. Functional proteins are drawn as rectangles, non-functional proteins as star-shapes.

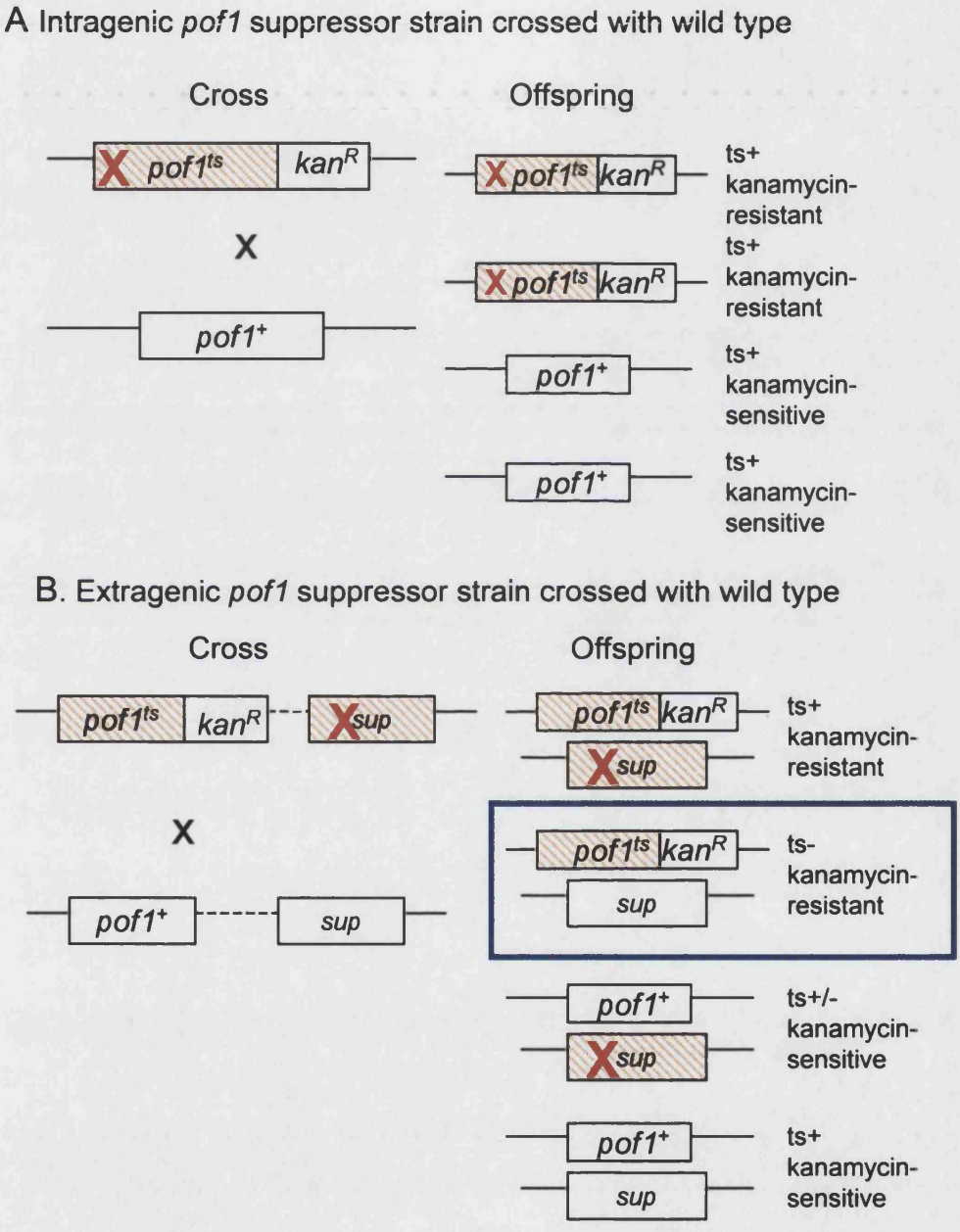


Figure 4.2 Illustration of method to determine if suppressor mutation is intragenic or extragenic to *pof1* locus.

A. If mutation is intragenic crossing cells with a wild type strain will result in no temperature sensitive (ts-) progeny. **B** If mutation is extragenic, shown here by gene 'sup', temperature sensitive, kanamycin resistant progeny will be recovered. It is possible that the suppressor mutation itself will cause a ts phenotype but these cells can be distinguished from *pof1* ts as they are kanamycin sensitive.

None-wild type genes are shaded orange. Location of suppressor mutation is shown by red X

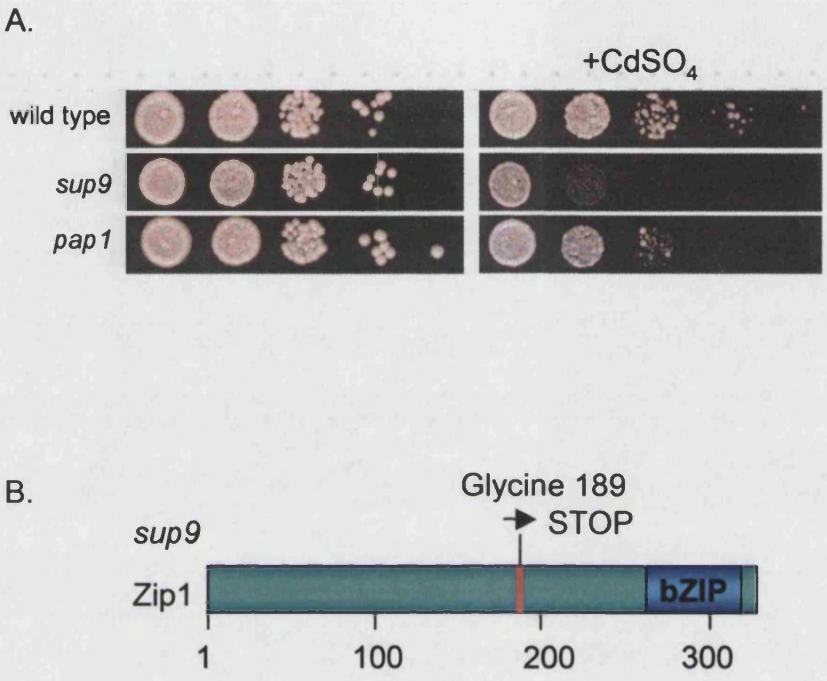


Figure 4.3 Identification of *zip1* as a extragenic suppressor locus of *pof1* mutants.

A Cadmium-hypersensitivity of *sup9* (*zip1*). Wild type (top row), *sup9* (=*zip1*, second), or *pap1*-deleted cells (bottom) were spotted onto rich plates in the absence (left) or presence (right) of 0.5 mM cadmium sulphate (10^5 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightwards) at 26°C and incubated for 4 d. **B** Schematic structure of Zip1. The position of the nonsense mutation in *sup9* (from glycine to TGA) and the C-terminal ZIP region are shown.

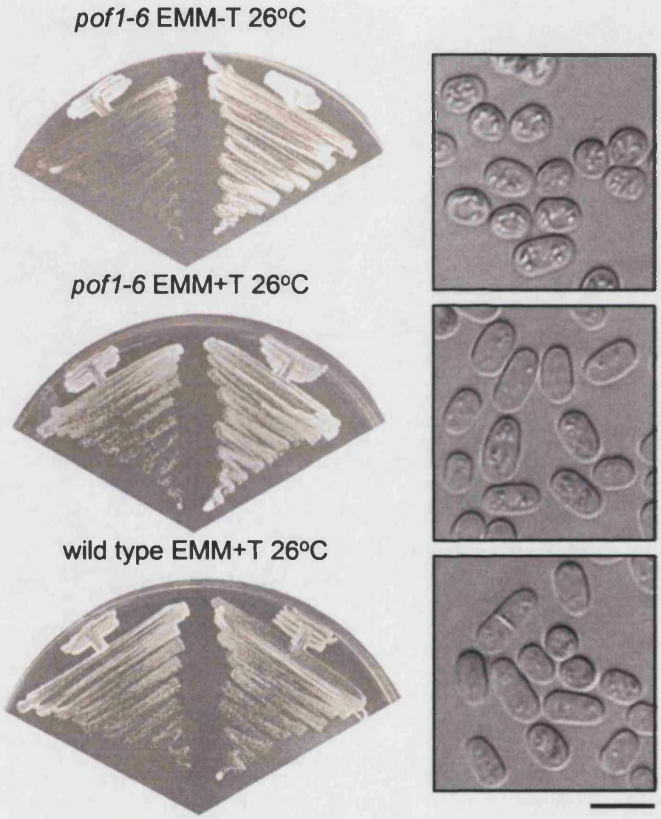


Figure 4.4 *zip1*$^+$ overexpression is toxic to *pof1-6* cells at non-restrictive temperature.

Toxicity of mildly overexpressed *zip1*$^+$. Plasmids containing *zip1*$^+$ under the thiamine repressible *nmt41* promoter (left) or empty vector (right) were transformed into *pof1-6* (top and middle plates) or wild type cells (bottom) and streaked on minimal plates in the absence (top and bottom) or presence (middle) of thiamine and incubated at 26˚C for 3 d. Morphology of cells containing *nmt41-zip1*$^+$ under each condition is shown in the righthand panels. The bar indicates 10 μm.

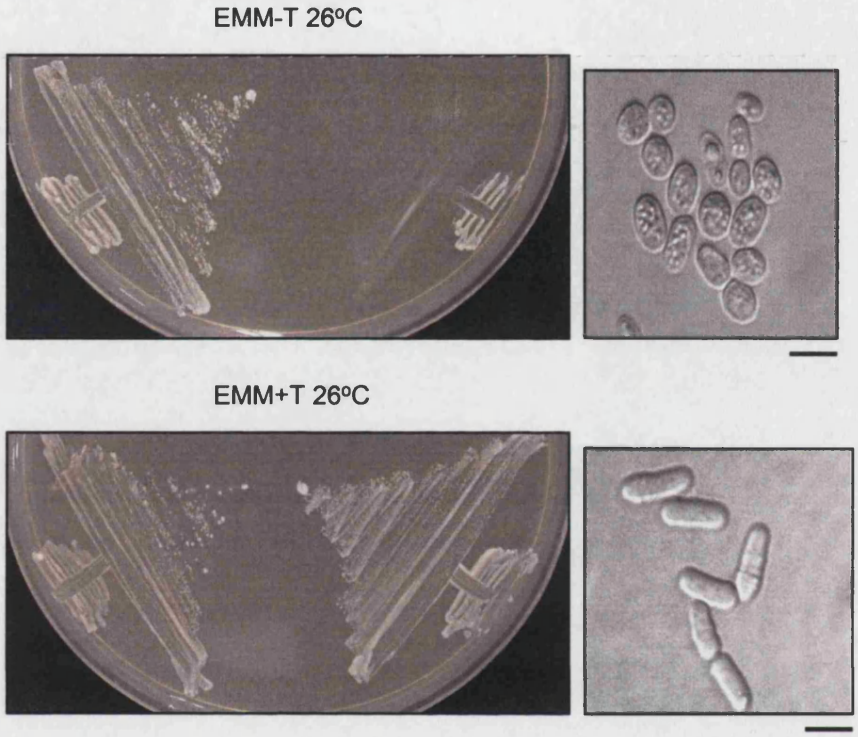


Figure 4.5 *zip1*$^{+}$ overexpression is toxic to wild type cells.
Toxicity of strongly overexpressed *zip1*$^{+}$. Strains containing *zip1*$^{+}$ under the control of the endogenous (lefthand side) or thiamine repressible *P3nmt* (righthand side) promoter were patched onto minimal media plates in the absence (top) or presence (bottom) of thiamine and incubated at 26˚C for 3 d. Morphology of cells containing *P3nmt-zip1*$^{+}$ under each condition is shown in the righthand panels. The bar indicates 10 μm.

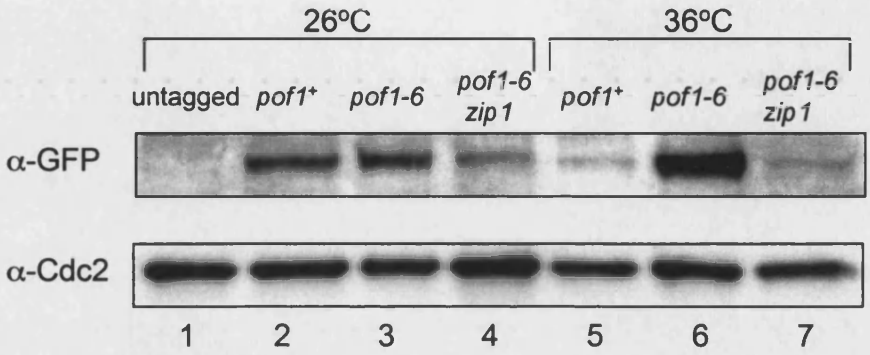


Figure 4.6 Pof1 accumulation in *pof1* ts mutants is *zip1*$^+$ dependent.

pof1$^+$-GFP (lanes 2 and 5), *pof1-6*-GFP (lanes 3 and 6) and *pof1-6*-GFP *zip1* (lanes 4 and 7) strains were cultured to exponential phase then grown for 4h at 26°C (lanes2-4) or 36°C (lanes 5-7). Protein extracts were made and immunoblotting performed using anti-GFP(upper panel) or anti-Cdc2 (lower panel) antibody. An untagged strain was run as a control (lane 1).

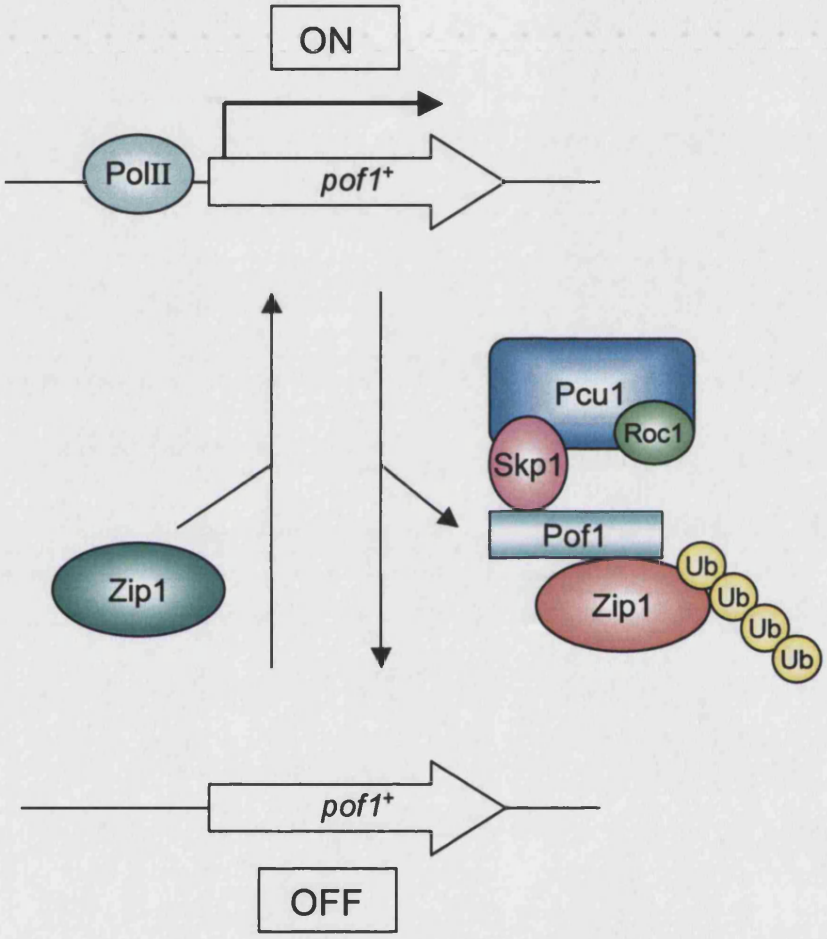


Figure 4.7 A model of the potential feedback loop operating between Pof1 and Zip1 activities.
Active Zip1 (green) leads to *pof1*$^{+}$ transcription, increase in SCF^{Pof1} activity and a subsequent inactivation of Zip1 (red). In turn this leads to a reduction in *pof1*$^{+}$ transcription, reduced SCF^{Pof1} activity and an increase in Zip1 activity.

Chapter 5

The mechanisms by which Pof1 regulates bZIP transcription Factor Zip1

Introduction

Genetic data described in the previous chapter suggests that Zip1 overactivity leads to the phenotype seen in *pof1* temperature sensitive mutants. This indicates that Pof1 may be responsible for the downregulation of Zip1 activity. In this chapter the relationship between Pof1 and Zip1 will be further explored. Zip1 is shown to be ubiquitylated *in vivo*. The stability of Zip1 is shown to be Pof1-dependent and Zip1 is shown to directly interact with Pof1. The role of Pof1 in Zip1 ubiquitylation and the subsequent role of this ubiquitylation in regulation of Zip1 activity will be discussed.

5.1 Zip1 is ubiquitylated *in vivo*

The isolation of a *zip1* nonsense mutation as a suppressor of *pof1-6* mutants suggested that Zip1 overactivity was the cause of the *pof1-6* temperature sensitive phenotype. Since Pof1 is an F-box protein it was likely that this was because Zip1 was a substrate of SCF^{Pof1}. If this were the case it should be possible to find ubiquitin-conjugated forms of Zip1 in the cell. Since in many cases the ubiquitylation of a protein leads to rapid degradation it is often difficult to detect ubiquitylated substrates. To avoid this problem *mts3-1*, a temperature sensitive proteasome mutant (which can be detected by its distinctive elogated, swollen phenotype at 36°C) which is unable to degrade ubiquitin conjugates at the restrictive temperature was used (Seeger, M. et al., 1996). Zip1 was C-terminally tagged with a triple haemagglutinin (HA) protein epitope as described previously (see Appendix A for primer sequences and location). A strain containing both the *mts3-1* mutation and the Zip1-HA tag was selected by crossing the two strains, selecting kanamycin resistant spores and then identifying the temperature sensitive colonies by replica-plating to 36°C. In order to isolate ubiquitin conjugates this strain was transformed with a multicopy plasmid in which ubiquitin was tagged with a hexa histidine (HIS) epitope. The cells were grown to exponential phase then shifted to 36°C for one hour to inactivate *mts3.* Ni^{2+} chelate resin, which binds HIS epitopes, was used to pull down all ubiquitylated conjugates from cell extracts and the resulting precipitate blotted with anti-HA antibody to detect the presence of Zip1-HA (Figure 5.1). A characteristic smear pattern was seen in the His-Ubi lane, which was absent from the control lane (no tagged ubiquitin). This suggested that Zip1 existed as a polyubiquitin conjugate in cells.

The next obvious question to ask was whether the formation of the Zip1-ubiquitin was dependent upon Pof1. The experiment needed to be repeated

in a *pof1-6* background. In order to do this the *pof1-6* strain was crossed with the *mts3-1* mutant carrying Zip1-HA and tetrad dissection carried out. Colony PCR and temperature sensitivity were used to screen the tetrad colonies to identify the correct strain. This strain was, however, extremely slow growing even at 26°C and practically impossible to propagate (Figure 5.2, upper). Examination of these cells from plates showed that they displayed an identical phenotype to *pof1*-6 at the restrictive temperature, small-sized cells (Figure 5.2, lower). Although this meant the dependency of Zip1 ubiquitylation upon Pof1 could not be directly addressed, this data in itself strongly implies that Pof1 and the proteasome are acting additively to inactivate the same substrate, Zip1.

5.2 The stability of Zip1 is *pof1*$^+$-dependent

I next asked whether the stability of the Zip1 protein is dependent upon Pof1 by examining the half-life of Zip1-HA in wild type and *pof1-6* cells. A Zip1-HA, *pof1-6* strain was constructed using an identical method to that described above for Zip1-HA, *mts3-1*. Exponentially growing cultures of both strains were shifted to 36°C for 2 hours then treated with protein synthesis inhibitor cyclohexamide (CHX) to shut off *de novo* protein synthesis. Samples of cells were taken at regular intervals, cell extracts prepared and Zip1 levels in these extracts examined by immunoblotting with anti-HA (Figure 5.3A, upper panels). It was clear from the initial time point (0 min) that *pof1*-6 cells have increased total Zip1 levels when compared to wild type cells (lane 2). In addition Zip1 in this mutant accumulated in several slower migrating forms, whilst in wild type cells only the fastest migrating of these forms was seen. Upon addition of CHX, in wild type, Zip1-HA disappeared quickly; after 15 min very little signal was detected (upper gel, lane 3). In clear contrast, in the *pof1-6* mutant the same faster migrating Zip1-HA band showed an increased half-life compared to wild type (lower gel and Figure 5.3B for quantification). A

band migrating slightly more slowly than this band also appeared to be stabilised in the *pof1-6* mutant (see lower gel, levels of this band are not quantified). However the most slowly migrating band seemed to be still degraded relatively efficiently in the *pof1-6* strain. Thus Pof1 is required for the instability of the form of Zip1 which is seen in exponentially growing wild type cells. Since there are some reports that the stability of an F-box protein depends upon its ability to recruit a substrate (Li, Y. et al., 2004), the stability of the Pof1 protein itself was also examined by immunoblotting with anti-GFP (Figure 5.3A, middle panels). Although as shown previously Pof1 was accumulating in the mutants at the first timepoint, subsequent levels following the CHX arrest decreased rapidly, comparable to the wild type situation, suggesting that Pof1 itself is not stabilised in the *pof1-6* mutants.

5.3 Zip1 is phosphorylated *in vivo*

The appearance of multiple Zip1 bands in the *pof1-6* mutant suggested that Zip1 was undergoing some kind of post-translational modification in this strain. As discussed previously, many F-box protein substrates are phosphorylated and this phosphorylation is required for their recognition by the SCF complex. Thus it was possible that some of these Zip1 bands corresponded to phosphorylated Zip1. To investigate this the *pof1-6* strain containing Zip1-HA was grown to exponential phase, shifted to 36°C for 4 hours and cell extract made. Zip1-HA was immunopurified from this extract using anti-HA, and the resulting precipitate treated with λ–phosphatase, an enzyme which removes phosphate groups from serine, threonine or tyrosine residues in proteins. For this experiment a gradient SDS-PAGE gel was used to analyse the extracts. This contains an acrylamide gradient so gives better resolution of protein bands. On this gel Zip1 appeared to exist as two clear bands in the *pof1-6* strain which both disappeared when treated with λ-phosphatase (Figure 5.4, see lanes 4 and 5). By comparing the molecular weights of these bands it appeared that the upper of the two corresponded to

the upper, non-stabilised band in the CHX experiment and the lower of the two corresponded to the upper band in the stabilised doublet. Thus Zip1 exists in two modified forms in *pof1-6* mutants, both of which are phosphorylated. One of these forms appears to depend upon Pof1 for stability but the other is degraded independently of Pof1.

5.4 Pof1 binds to Zip1 in wild type cells

If Zip1 is a substrate for SCF^{Pof1}, Pof1 is expected to bind Zip1. This would be expected to occur in a wild type strain but since Zip1 would be expected to be degraded rapidly following interaction with Pof1, an *mts3-1* background must again be used. Immunoprecipitation was thus performed in a strain containing Pof1-GFP and Zip1-HA in an *mts3-1* background. This strain was constructed by crossing the Zip1-HA, *mts3-1* strain described above with a Pof1-GFP strain. Tetrad analysis was carried out, the location of the Pof1-GFP and Zip1-HA spores identified using colony PCR, and *mts3-1* then identified by temperature-sensitivity. In an *mts3-1* background Zip1 was seen as a doublet, whose size correlated to the faster-migrating stabilised doublet seen in the half-life experiment in *pof1-6* cells (Figure 5.5, lanes 2 and 4, lower panel). Presumably the upper band of this doublet corresponds to phosphorylated Zip1 which cannot be degraded in an SCF^{Pof1}-dependent manner due to the inhibition of the proteasome in this strain. Immunoprecipitation using anti-GFP antibody showed that Pof1 co-immunoprecipitated with Zip1, specifically the upper band of the doublet (Figure 5.5, compare lanes 4 and 5, lower panel). Reciprocal immunoprecipitation with anti-HA antibody also co-precipitated Pof1-GFP, albeit very faintly (lane 4). This data suggests that Pof1 specifically interacts with the phosphorylated form of Zip1 *in vivo*.

5.5 Comparison of binding between Pof1 and modified forms of Zip1

In the *pof1-6* mutant two phosphorylated forms of Zip1 were seen to accumulate. One of these forms (the slowest migrating band) appeared not to be stabilised in the *pof1-6* mutant (Figure 5.3). This fact suggested that this form of Zip1 was not normally degraded by SCF^{Pof1}. To investigate this idea further the interaction of Pof1 with different Zip1 bands in a *pof1-6* strain was investigated using immunoprecipitation. A *pof1-6* and *pof1*$^{+}$ strain, both of which contained Pof1-GFP (as explained previously this was mutant Pof1-GFP in the *pof1-6* strain) and Zip1-HA were used. The *pof1-6, mts3-1* Zip1-HA strain was constructed using tetrad analysis and colony PCR as described above. The wild type *pof1*$^{+}$ strain contained an *mts3-1* mutation, so that the binding between wild type Pof1 and Zip1 could be used as a control experiment. It was clear from total extracts from these strains that the slow migrating Zip1 band did indeed accumulate in the *pof1-6* mutant but not the *mts3-1* mutant (Figure 5.6, compare lanes 2 and 3, lower panel). Immunoprecipitation with anti-HA antibody co-precipitated small amounts of Pof1 in both strains (Figure 5.6, lanes 4 and 6). Reciprocal immunoprecipitation with anti-GFP antibody showed only the slow mobility form of Zip1 co-precipitating in the *mts3-1* strain, as in the previous experiment (Figure 5.6, lane 5). In the *pof1-6* strain both of the slower migrating Zip1 bands appeared to co-precipitate with Pof1, suggesting the slowest migrating Zip1 band could in fact interact with Pof1 (Figure 5.6, lane 7). However the two Zip1 bands which co-precipitated with Pof1 in the *pof1-6* strain were of similar intensity, but in the total extract the upper band was much stronger (Figure 5.6, compare lanes 3 and 7, lower panel). This suggests that although the upper band can interact with Pof1 it may do so with reduced efficiency compared to the other modified form of Zip1.

Summary

This chapter has described the experiments carried out to determine how Zip1 is regulated in the cell and whether this regulation is Pof1-dependent. Zip1 has been shown to be multi-ubiquitylated in a proteasome mutant. It could not be shown if this was Pof1-dependent. However Zip1 accumulates in *pof1* mutants and some forms of it are stabilised, suggesting Pof1 is necessary for Zip1 degradation. Zip1 is also able to exist in two phosphorylated forms. Only one of these forms is seen in *pof1*$^{+}$ cells and seems to be required for interaction with Pof1. The other phosphorylated form is only seen in *pof1-6* mutants but is not stabilised in this strain. It is also able to interact with Pof1 in these conditions, however this interaction may be less efficient than the other modified form.

Discussion

The *S. cerevisiae* homologue of Pof1, Met30, is known to negatively regulate its substrate, Met4, by degradation-dependent and independent mechanisms (see Chapter 1). Met30 is known to be required for Met4 ubiquitylation, and this ubiquitylation generally switches off Met4 but does not lead to its instability, although it has been reported that ubiquitylated Met4 can be degraded in certain growing conditions (see Chapter 1). The experiments described in this chapter were carried out to try and ascertain if similar mechanisms existed between Pof1 and Zip1.

The fact that a nonsense allele of *zip1* was isolated as a suppressor of *pof1-6* suggested that *pof1-6* cells arrested growth due to an excess of Zip1 activity. This would indicate that Zip1 was a substrate of SCF^{Pof1}. This idea is further supported by experiments described in this chapter for the following reasons. Firstly, Zip1 is shown to be ubiquitylated *in vivo*. Although the role of Pof1 in this process could not be directly addressed, the fact that Zip1 is ubiquitylated means it must be the substrate of some ubiquitin ligase. Secondly, Zip1 is

phosphorylated, a modification known to be used to regulate recognition of substrates by SCF complexes, and thirdly, this phosphorylated form is able to interact with Pof1. The interaction of Zip1 with Pof1 is strong evidence that it is a Pof1 substrate.

The data presented in this chapter would also suggest that unlike Met4, Zip1 interaction with Pof1 leads to its degradation, rather than just inhibiting its activity. This is because firstly, Zip1 is seen to accumulate in *pof1-6* mutants, suggesting that *pof1*$^{+}$ normally negatively regulates Zip1 levels. Secondly *mts3-1 pof1-6* is toxic and the cells arrest with a phenotype identical to *pof1-6* at restrictive temperature, suggesting that Pof1 and the proteasome usually act together to inhibit the same substrate and thirdly, *pof1-6* mutants contain a stabilised form of Zip1 in the halflife experiments. Thus it is likely that Pof1 interacts with Zip1 and this leads to the degradation of Zip1 by the proteasome, presumably through the ubiquitylation of Zip1. The fact that ubiquitylated forms of Zip1 are only seen in proteasome mutants, in direct contrast to Met4 which is found to exist in an ubiquitylated state in normal growing conditions (Flick, Ouni et al. 2004), suggests that proteolysis independent ubiquitylation of Zip1 does not occur.

The most intriguing aspect of Zip1 regulation concerns the function of the second phosphorylated Zip1 species, seen to accumulate as a slower migrating band in *pof1-6* cells. This band is not seen to accumulate in a proteasome mutant or be stabilised in a *pof1-6* mutant. This suggests that it is not directly ubiquitylated by Pof1 and sent to the proteasome for degradation. If this is the case why does it accumulate in a *pof1-6* mutant? A possible explanation is that this phosphorylated species is made by default for some reason if non-ubiquitylated Zip1 levels increase. This model is described in Figure 5.7. It could be speculated that in wild type cells Zip1 is made, phosphorylated and ubiquitylated by Pof1 before this other phosphorylation

can occur, thus this phosphorylated form of Zip1 is never observed. *mts3-1* mutants presumably have increased Zip1 levels but since Pof1 is present this is probably ubiquitylated, thus again does not get modified. However *pof1* mutants have increased amounts of non-ubiquitylated Zip1 present, and this other form of phosphorylation happens by default. This is only a model and in itself raises many questions. Firstly, what is the function of this other phosphorylated form of Zip1. Possibly this form is required for a specific transcriptional response, for example to stress or other special conditions. Under these conditions the cell could switch off Pof1-dependent ubiquitylation of Zip1 and the phosphorylated form would be made by default and allow a rapid transcriptional response. The second question concerns how this form is itself regulated. It rapidly disappears following CHX arrest in *pof1-6* cells, thus it has a short halflife independently of Pof1. There are two possibilities to explain this fact. Either it is ubiquitylated by some other ubiquitin ligase complex, leading to its degradation, or it can be rapidly dephosphorylated and that is why it seems to disappear following CHX arrest. If the latter were true one would predict that levels of the non-phosphorylated band should actually increase following CHX arrest and this does not occur so the Pof1 independent degradation of the phosphorylated Zip1 species seems a more likely explanation for its instability.

Whether the phosphorylated form of Zip1 interacts *in vivo* with Pof1 remains unclear from the immunoprecipitation experiments. It seems to co-precipitate with Pof1 but this may not be highly efficient. In any case the fact that this form is not stabilised in a *pof1* mutant suggests even if it does interact with Pof1 this interaction may not result in the ubiquitylation of this form of Zip1, thus the interaction shown in Figure 5.6 may have no physiological consequence for the cell.

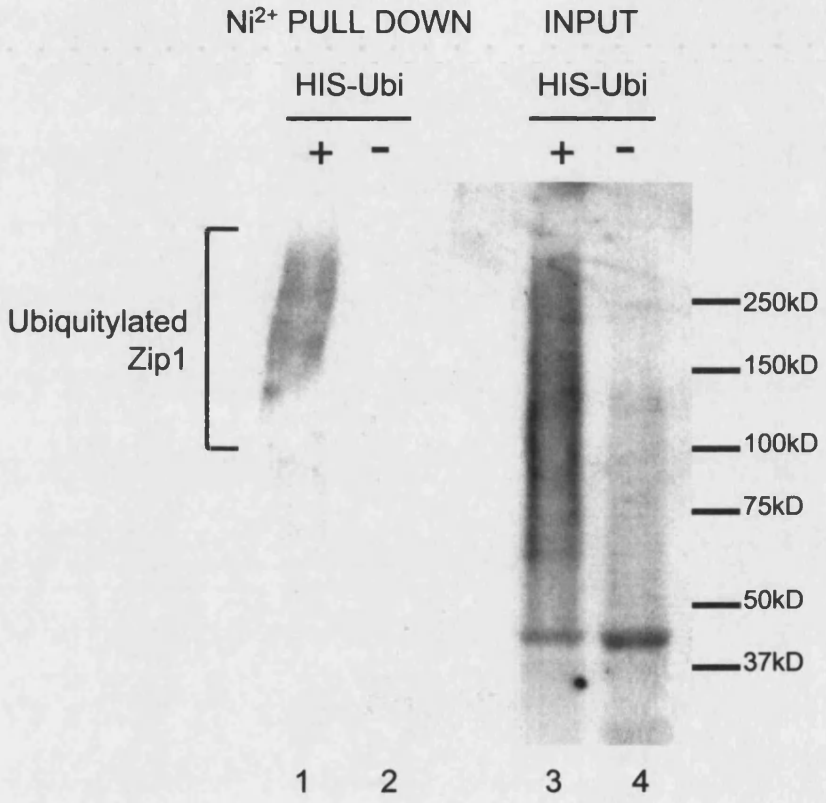


Figure 5.1 Zip1 is polyubiquitylated in vivo.

Plasmids containing 6His-ubiquitin (His-Ubi, lanes 1 and 3) or empty vector (lanes 2 and 4) were introduced into the *mts3-1* mutant containing Zip1-HA. After 1 h incubation at 36°C, ubiquitylated proteins were purified with Ni^{2+}-NTA beads (lanes 1 and 2). Total extracts (60 μg) are also run (lanes 3 and 4). Immunoblotting was performed with anti-HA antibody.

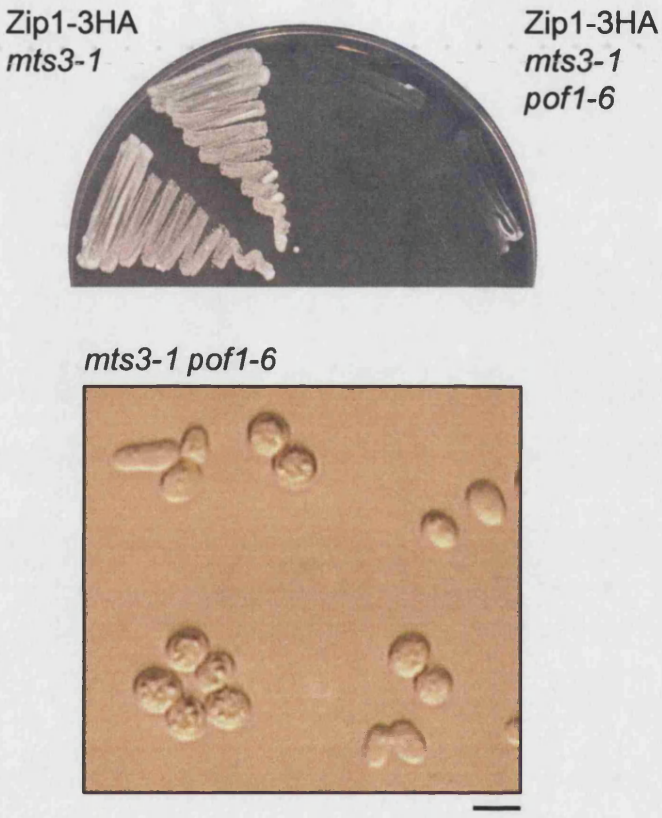


Figure 5.2 Synthetic phenotype between *pof1-6* and *mts3-1*.

mts3-1 single (left) or *mts3-1 pof1-6* double mutants (right) containing Zip1-HA were streaked on rich medium and incubated at 26°C for 3 d (upper). Cell morphology for *mts3-1 pof1-6* is also shown (lower). The bar indicates 10 μm.

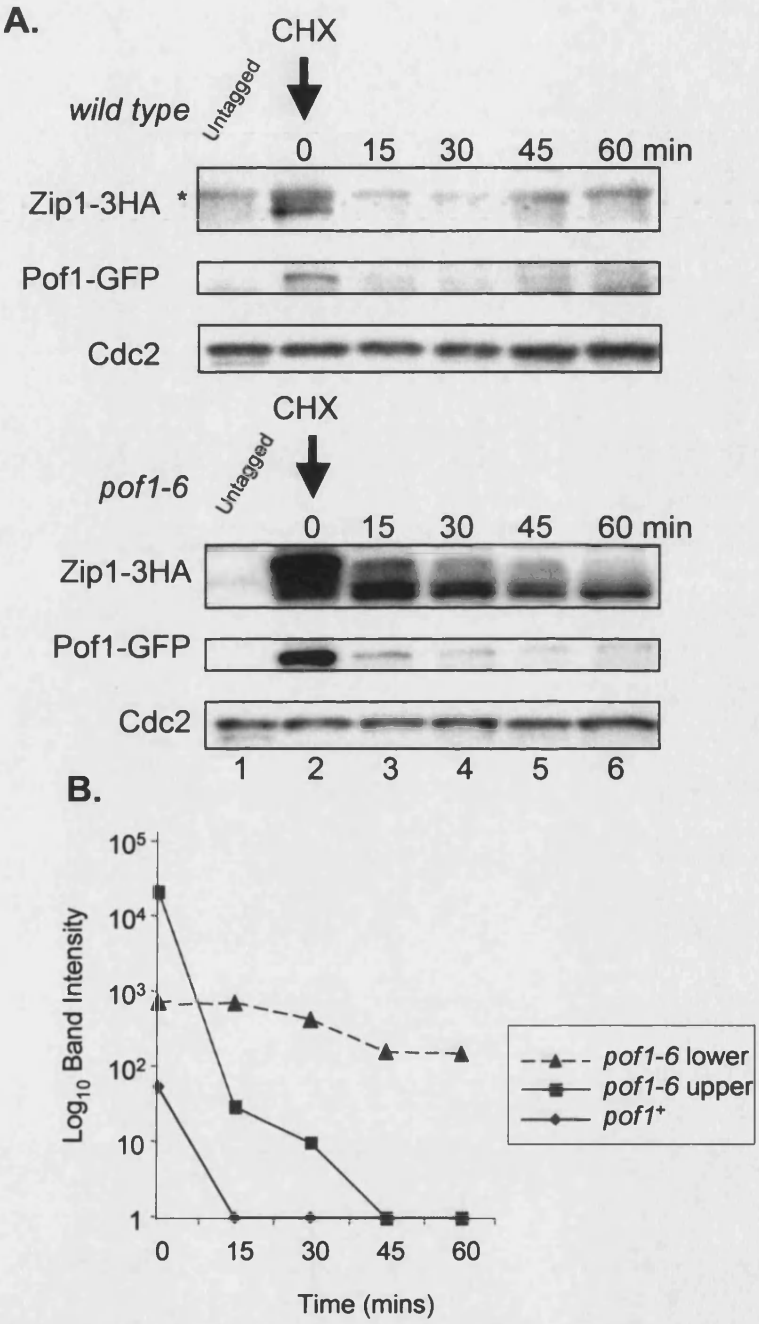


Figure 5.3 Zip1 is stabilised in *pof1* mutants.

(A) Accumulation and partial stabilisation of Zip1 in *pof1-6*. Exponentially growing wild type *pof1*$^{+}$-GFP (upper gel) or *pof1-6*-GFP (lower) cells containing Zip1-HA were shifted to 36°C for 2h and cyclohexamide (CHX, 100 μg/ml) was then added. Protein samples were prepared at indicated time points. Immunoblotting was performed with anti-HA (top), anti-GFP (middle) or anti-Cdc2 antibody (bottom). Samples from untagged strains were also run as a negative control (lane 1). (B) Quantification of Zip1 levels. Zip1-HA amount shown in (A) was quantified. Intensities of upper (circles), or lower band of Zip1-HA in *pof1-6* (squares) and a lower band in wild type (triangles) were measured.

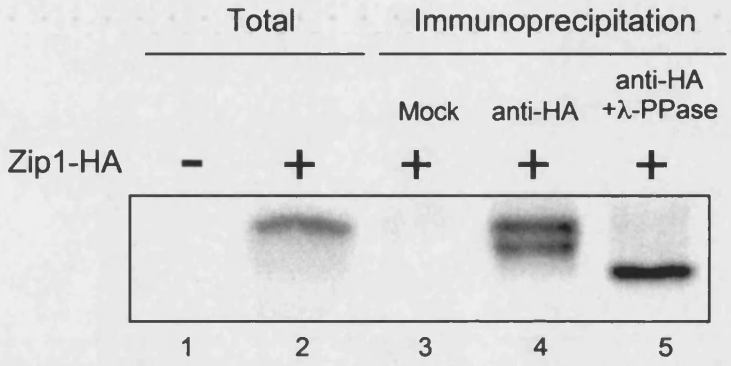


Figure 5.4 Zip1 is phosphorylated *in vivo*.
pof1-6 mutant cells containing Zip1-HA were shifted to 36°C for 4 h and immunprecipitaiton was performed with mock (lane 3) or anti-HA antibody (lanes 4 and 5). Immunoprecipitates were treated with λ-protein phosphatase for 30 min at 30°C (lane 5). Total extract (30 μg) was also run (lane 2). As a negative control protein samples from prepared from untagged *pof1-6* were also run (lane 1). Immunoblotting was performed with anti-HA antibody.

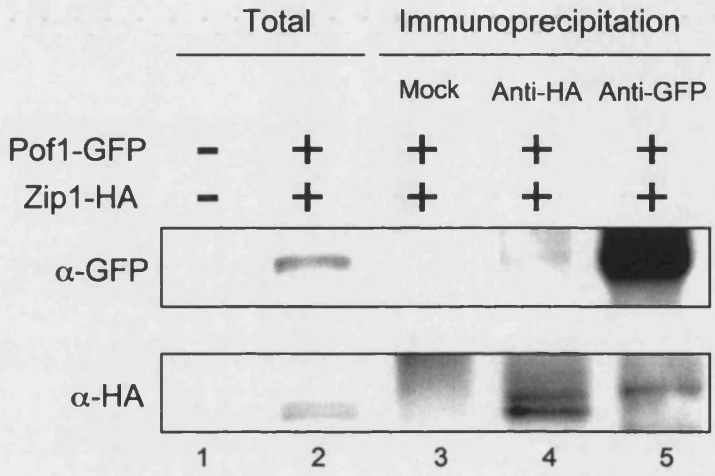


Figure 5.5 Binding between phosphorylated Zip1 and Pof1. Immunoprecipitation was performed with anti-HA (lane 4), anti-GFP antibody (lane 5) or mock (lane 3) using protein extracts prepared from *mts3-1* mutants containing Pof1-GFP Zip1-HA, which were incubated at 36°C for 1h. Total extracts from a tagged (lane 2) or untagged strain (lane 1) were also run. Immunoblotting was performed with anti-GFP (upper) or anti-HA antibody (lower).

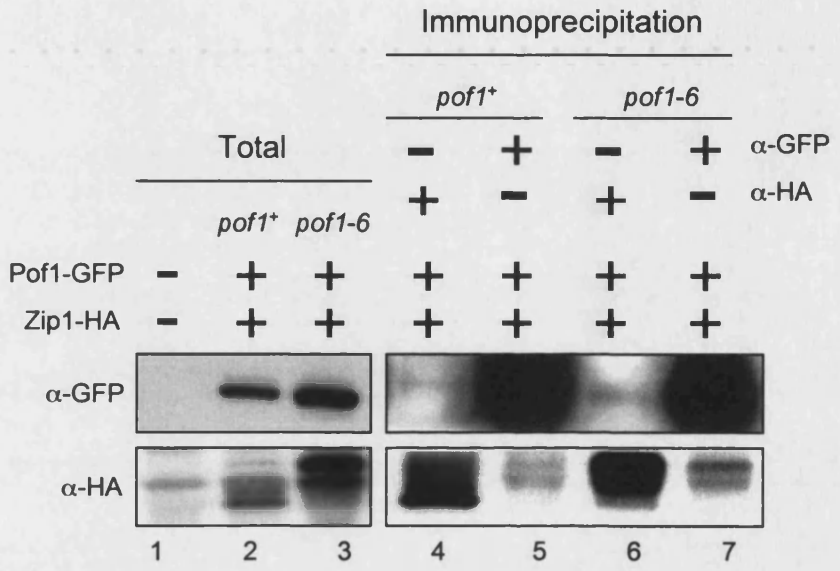


Figure 5.6 Comparison of binding between Zip1 and Pof1 in wild type and *pof1-6* mutants.

Immunoprecipitation was performed with anti-HA (lanes 4 and 5) or anti-GFP antibody (lanes 6 and 7) using protein extracts prepared from *mts3-1* (lanes 2, 4 and 5) or *pof1-6* (lanes 3, 6 and 7) mutants containing Pof1-GFP Zip1-HA, which were incubated at 36°C for 2h. Total extracts from tagged (lanes 2 and 3) or untagged strains (lane 1) were also run. Immunoblotting was performed with anti-GFP (upper) or anti-HA antibody (lower).

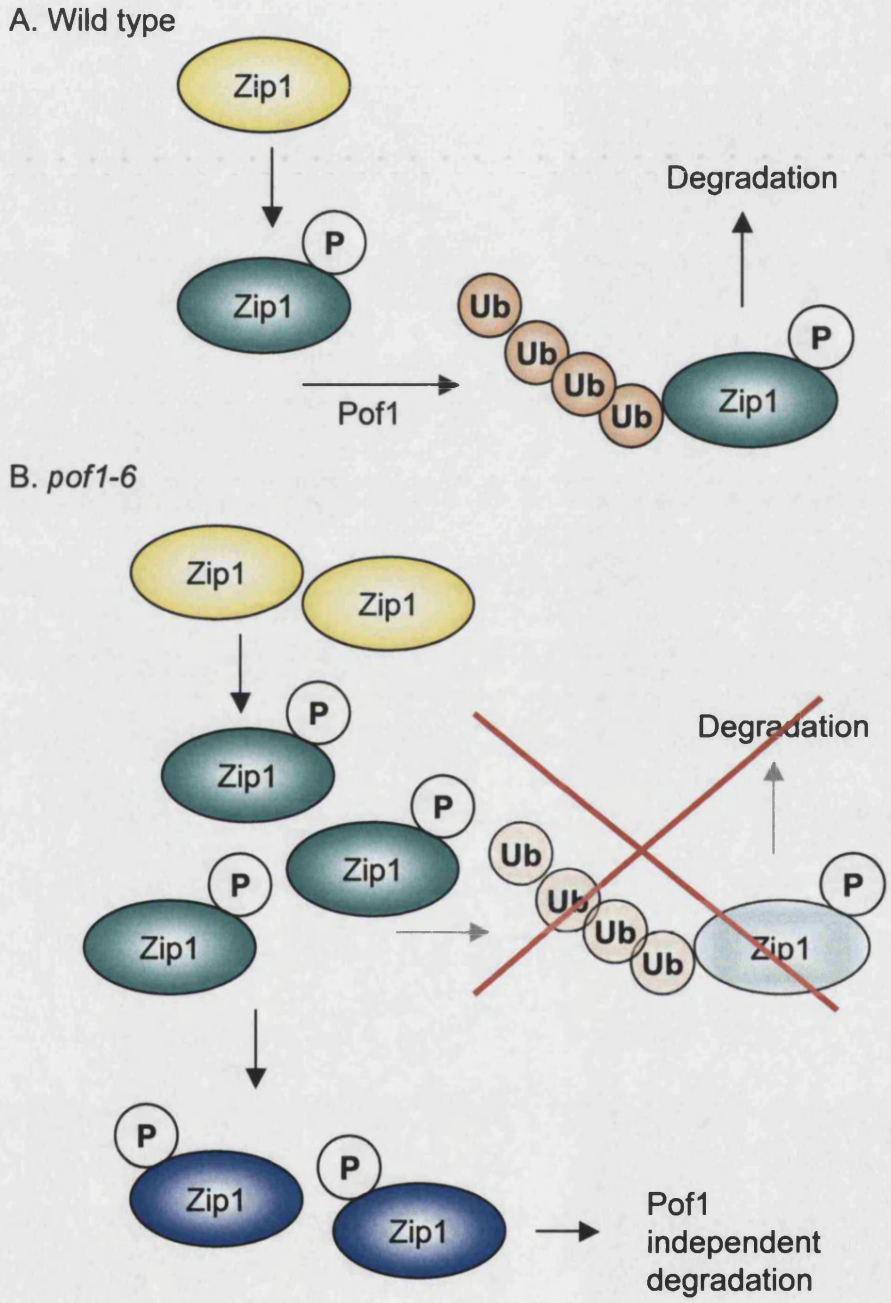


Figure 5.7. Model of Zip1 Regulation.
Three alternative forms of Zip1 are shown; unmodified (yellow); fast- moving phosphorylated (green) and slow-moving phosphorylated (blue). **A** In wild type cells Zip1 is rapidly ubiquitylated by SCF^{Pof1} and degraded before slow-moving phosphorylated band is produced. **B** In *pof1* mutants Zip1 cannot be ubiquitylated, both unmodified and fast moving phosphorylated band accumulate and slow moving phosphorylated band is made by default.
Fast and slow-moving refers to position of bands on an SDS-PAGE gel.

Chapter 6

The role of the Zip1 transcription factor in the cell

Introduction

Data described in previous chapters suggests that it is too much Zip1 activity which leads to the growth arrest and small cell phenotype seen in *pof1* mutants at restrictive temperature. This chapter will discuss how an excess of Zip1 activity could lead to this phenotype. It will begin with an analysis of the *in vivo* function of Zip1, in particular its role in cadmium stress. The identification of the genes transcribed by Zip1 in response to cadmium using microarray analysis will be described and the possibility that Zip1 is a cadmium-stress specific activator of the sulphate assimilation pathway will be discussed. The general physiological response of cells to cadmium will then be analysed and the possibility that the *pof1* phenotype is due to cells eliciting an overactive, Zip1-dependent cadmium stress response will be explored.

6.1 Zip1 is specifically sensitive to cadmium stress

As shown previously, a *zip1* nonsense allele is cadmium sensitive. It was possible *zip1*$^+$ was involved specifically in the cellular response to cadmium stress or was a core stress response gene required for the response of cells to many different types of stress. In order to test this the growth of the *zip1* nonsense allele was tested in response to many different types of stress; osmotic stress (potassium chloride, KCl); oxidative stress (tert-butylhydroperoxide, tBOOH); S-phase arrest (hydroxyurea, HU); DNA damage (methyl methanesulphonate, MMS); exposure to ultraviolet radiation (UV); microtubule-destabilising drugs (thiabendazole, TBZ); a sulphate analogue (sodium selenate); caffeine and a translational inhibitor, cyclohexamide. As controls strains with deletions of three core stress response genes were used: *sty1*, a stress activated MAP kinase, reported to be multidrug, osmotic stress, heat stress, oxidative stress, and UV irradiation-sensitive (Degols, G. and Russell, P., 1997; Degols, G. et al., 1996; Millar, J.B. et al., 1995; Shieh, J.C. et al., 1997; Shiozaki, K. and Russell, P., 1995), *pap1*, a transcription factor downstream of the Sty1 pathway, which is multidrug and oxidative stress sensitive and *crm1*, a negative regulator of Pap1, which is thus multidrug resistant(Adachi, Y. and Yanagida, M., 1989; Kumada, K. et al., 1996; Toda, T. et al., 1992; Toda, T. et al., 1991; Toone, W.M. et al., 1998). When compared to wild type cells *zip1* was sensitive to tBOOH, UV irradiation, sodium selenate, caffeine and cyclohexamide (Figures 6.1 and 6.2). However in all cases except sodium selenate, *zip1* was not as sensitive as *pap1* (tBOOH, caffeine and cyclohexamide) or *sty1* (UV irradiation). In the case of sodium selenate *zip1* was equally as sensitive as *sty1* and *crm1*. In contrast, as shown in Chapter 4 (Figure 4.3), when exposed to cadmium stress *zip1* was far more sensitive than *pap1*. This suggests that the primary role of *zip1*$^+$ is in cadmium stress response. Many factors

involved in stress responses are shared between different types of stress. Glutathione, for instance, is used to protect cells against cadmium and oxidative stress. The minor sensitivity of *zip1* to stresses other than cadmium is likely to be due to fluctuations in these kinds of factors rather than a direct effect.

6.2 Identification of downstream targets of Zip1 using microarray analysis

Since Zip1 was a bZIP transcription factor it was likely to be involved in altering the transcriptional response of the cell to cadmium stress. DNA microarrays can be used to detect changes in global gene expression profiles in response to stress; cells can be grown in the absence or presence of some stress, RNA extracted from both cultures, labelled through reverse transcription and the resulting cDNA hybridised onto DNA microarrays containing probes for the fission yeast genome. The microarrays are scanned and differences in gene expression between the two conditions can be calculated. In order to study Zip1-dependent gene expression a microarray experiment was carried out as follows; the *zip1* nonsense allele that was initially isolated as a *pof1-6* suppressor was crossed into a prototrophic wild type background. This means it contained no nutritional markers. This is useful for microarray studies as it makes analysis of the resulting data easier since strains containing mutations in such markers need nutrients adding to the growth media and this leads to complicated patterns of gene expression which it is simpler to avoid. A prototrophic wild type and the *zip1* strain were grown to exponential phase and then exposed to 0.5mM cadmium sulphate, cells were harvested at 0, 15 and 60 minute timepoints, RNA extracted and levels of gene expression measured using microarrays as described above. This experiment and the subsequent data analysis were carried out by Dr D.

Chen in the laboratory of Dr J.Bahler at the Sanger Centre, Cambridge, UK. Data for 3200 genes was generated and initial analysis carried out. This resulted in a list of 57 genes which showed some difference in expression between wild type and *zip1* strains either at 0 minutes, thus were likely to rely on Zip1 for basal expression, or at 15 or 60 minutes, thus were likely to rely on Zip1 for cadmium induced transcriptional changes. All expression data had been normalised against that of wild type at 0 minutes, so it was easy to see if gene expression had gone up or down. From this data I identified 27 genes whose expression in wild type cells and *zip1* cells varied by two-fold or greater at any one of the three timepoints (Table 5 shows the function of these genes and Table 6 shows the normalised expression data). Thus this list should contain any genes that are transcribed (or repressed) by Zip1 either in the absence or in response to cadmium stress. In actual fact no genes appeared to require Zip1 for basal level expression only. Only four genes showed a two-fold or greater decrease in expression in the *zip1* strain at 0 minutes compared to wild type. These were C869.05c; C1739.06c; PB10D8.02c and PB10D8.01 (see Table 6) and all of these showed significant *zip1*-dependent cadmium induction. Thus the first conclusion to arise from the microarray data is that Zip1 transcription mainly occurs in response to cadmium and not in a normally growing cell. However it is important to note that many genes had altered expression in a *zip1* strain but failed to make the two-fold cut-off, thus it is possible there are some less severe transcriptional changes in a *zip1* cell even in the absence of cadmium, and these may well have some minor effect on the cell. Although the *zip1*-delete appeared phenotypically wild type it was noted to grow slightly more slowly than wild type cells on plates and this could be due to a lack of such basal level transcription.

Zip1-dependent genes

The 27 genes identified as requiring Zip1 for transcription fall into several

clear categories. Firstly, there are many genes whose products are involved in the sulphate assimilation pathway. This pathway is required for the production of sulphur amino acids and glutathione and, as described in Chapter 1, is known to be upregulated in budding yeast in response to cadmium stress through the homologue of Zip1, Met4. However compared to the number of *MET* genes known to be regulated by Met4 (Thomas, D. and Surdin-Kerjan, Y., 1997) far fewer of the *S. pombe* sulphate assimilation pathway genes appear to be transcribed by Zip1. Secondly there are several genes whose products appear to be membrane transporters of some kind. This is in agreement with previous reports of the transcriptional responses of cells to cadmium stress, which have suggested sulphur, zinc and GSH transporters are all upregulated in response to cadmium. Thirdly, Several genes whose products are involved in metabolic processes were induced. Although it is difficult to say exactly why these genes are required following cadmium exposure, this stress obviously causes a lot of metabolic problems in the cell, for instance glutathione depletion would lead to oxidative stress. So it is understandable that some changes in the transcription of genes such as flavin-dependent monooxygenases may occur. Finally, there are six genes which do not seem to fit into any of the above categories; *cdc15*$^{+}$, a gene required for septation and cytokinesis; *cdc22*$^{+}$, the large subunit of ribonucleotide reductase which is involved in nucleotide production for DNA synthesis; three genes of unknown function, two of which (C1348.06c and C977.05c) are known to be expressed in response to zinc depletion and finally *pof1*$^{+}$, which supports the idea that Zip1 upregulates *pof1*$^{+}$ transcription as a feedback mechanism, as discussed previously. Apart from *pof1*$^{+}$ it is not obvious why any of these genes would be upregulated in response to cadmium stress.

Cadmium-specific, Zip1-dependent genes

From the 27 genes which appear to require Zip1 for transcription, 19 were expressed more than two-fold in response to cadmium in wild type cells. These are highlighted in bold in Tables 5 and 6. This suggested that Zip1 had a role in the induction of genes in response to cadmium stress. Global analysis of gene expression in response to stress has revealed that two different groups of genes are expressed following exposure to a specific stress, the core environmental stress response genes (CESR), which are commonly expressed following most stresses, and stress specific genes, which vary depending upon the stress encountered (Chen, D. et al., 2003). A group of 32 genes has been defined which are expressed specifically in response to cadmium stress (Chen, D. et al., 2003). To establish whether the Zip1-dependent genes expressed in response to cadmium were CESR or cadmium-specific genes, the list of 19 Zip1-dependent genes was compared to the 32 cadmium specific genes. As illustrated in Figure 6.3, 14 of the Zip1-dependent genes expressed in response to cadmium are cadmium specific genes. Taken together these data suggest that the primary role of Zip1 is to transcribe genes as a specific response to cadmium stress.

Cadmium-specific, Zip1-dependent genes are expressed in a *sty1*$^{+}$ independent manner

As described above Sty1 is a protein kinase involved in the regulation of numerous stress responses. There are two known downstream targets of Sty1: Pap1, a transcription factor involved in multidrug and oxidative stress resistance (see above) and Atf1, a transcription factor involved in the osmotic stress and nutrient deprivation response (Kanoh, J. et al., 1996; Shiozaki, K. and Russell, P., 1996; Wilkinson, M.G. et al., 1996). Microarray studies of global transcriptional responses to stress have been carried out in wild type and *sty1* or *atf1*-deleted strains thus it is known in wild type cells if the transcription of a gene in response to a specific stress is Sty1 or Atf1-

dependent (Chen, D. et al., 2003). To see if it is likely that Zip1 is acting downstream of the Sty1 pathway the list of 14 Zip1-dependent cadmium specifically induced genes was compared to this wild type data. As shown in Table 7 only one of the Zip1-dependent genes requires Sty1 for induction in response to cadmium. Thus it is likely that Zip1 is responsible for a cadmium specific response independently of the Sty1 pathway.

6.3 Zip1 accumulates in a modified form in response to cadmium exposure

If Zip1 transcriptional activity was part of the cellular response to cadmium stress it was likely that the activity of Zip1 would need to be increased following exposure to this stress. This could be accomplished by increasing Zip1 levels in the cell, potentially by inhibiting Pof1-dependent Zip1 ubiquitylation. To investigate this idea, total Zip1 levels following cadmium exposure were measured. Strains containing Zip1-HA were grown to exponential phase, treated with cadmium sulphate and samples taken at 0, 1 and 4 hours. Extracts were run on an SDS-PAGE gel and immunoblotted with anti-HA antibody. As seen in Figure 6.4, after only 1 hours exposure to cadmium, Zip1 levels had significantly increased and this was even more noticeable after 4 hours. In addition Zip1 was accumulating in a modified form, which appeared to be a similar molecular weight as the slow-migrating band seen to accumulate in *pof1* mutants. Since no increase in $zip1^{+}$ transcription is seen in response to cadmium (Chen, D. et al., 2003), this accumulation of Zip1 is likely to be caused by repression of Zip1 degradation.

6.4 Zip1 is not essential for the sulphate assimilation pathway

The sulphate assimilation pathway is the process used by cells to produce organic sulphur compounds, such as sulphur amino acids, from inorganic sulphur sources (as described in Chapter 1). In nutrient-rich growing conditions there are usually plenty of organic sulphur compounds around and cells do not need to utilise this pathway, however in low nutrient conditions this pathway becomes essential for growth. In budding yeast Met4 transcribes many of the genes in this pathway, specifically for the production of methionine and cysteine when these are missing from the environment. Thus *met4*–deleted cells cannot grow without an external source of methionine. Several of the genes which, from the microarray data, appeared to be Zip1-dependent, are involved in this pathway. Although many of them appeared to rely on Zip1 for cadmium-induced transcription it was possible that Zip1 was also used in cadmium-free conditions to transcribe these genes. In particular, two of the four genes which showed a two-fold reduction in basal level transcription in a *zip1* strain, C869.05c and C1739.06c, are known components of this pathway. Thus the role of Zip1 in this pathway was investigated by testing whether cells deleted for $zip1^{+}$ could grow on media lacking an organic sulphur source. The *zip1*Δ strain was patched onto plates containing only an inorganic or an organic sulphur source, sodium sulphate or methionine respectively. As controls a wild type strain and a strain containing a mutation in a gene in the sulphur assimilation pathway were patched onto the same plates. As can be seen from Figure 6.5, unlike the *met5* mutant, *zip1*Δ could grow well on plates containing only an inorganic sulphur source. This suggests that while Zip1 is capable of upregulating some of the genes in the sulphur assimilation pathway in response to cadmium stress, it is not essential for the cells to synthesise sulphur amino acids under conditions where these amino acids are lacking.

6.5 Cells exposed to cadmium show some similar phenotypes to a *pof1* mutant and these are Zip1-dependent

Previous results suggested that Zip1 is specifically required to initiate a cadmium stress response. *pof1* mutants show an accumulation of Zip1 in a modified form, which is similar to the accumulation of Zip1 seen in cadmium exposed cells, and it appeared that it was this increase in Zip1 levels which lead to the *pof1* mutant phenotype. It was possible that the growth arrest and small cell size of *pof1* mutants was the result of a cadmium stress response being inappropriately activated, i.e. *pof1* mutants were acting like cells permanently exposed to cadmium. If this was the case, wild type cells exposed to cadmium should show some of the phenotypes seen in a *pof1* mutant but these phenotypes should be *zip1*$^{+}$-dependent. This idea was investigated by exposing wild type, *pof1-6* and *zip1*-deleted cells growing in culture to cadmium stress and observing their response (Figures 6.6. 6.7 and 6.8).

Cells exposed to cadmium retain viability in a Zip1-dependent manner

As shown in Chapter 2 although *pof1-6* cells arrest growth at restrictive temperatures they retain constant viability even after six hours, suggesting they do not actually die at these temperatures but simply halt division. It was possible that this is a response cells normally make when exposed to cadmium stress, so the ability of cells to retain viability when exposed to cadmium was investigated. 0.5 mM cadmium sulphate stopped growth of all cells at 26°C in liquid medium. However if samples of these liquid cultures were plated onto cadmium-free plates, even after 6 h exposure to cadmium, wild type and *pof1*-6 cells retained high viability (Figure 6.6). This implies that the growth arrest observed in these two strains is reversible. In sharp contrast, *zip1* cells lost viability very rapidly, with only 8% of cells surviving after two hours in the presence of cadmium. This indicates that the apparent

growth arrest in wild type and *pof1-6* cells upon cadmium stress is not due to cell death, instead that cells arrest growth but remain viable. Importantly this response is Zip1-dependent.

Cells exposed to cadmium become reduced in size in a Zip1-dependent manner

At restrictive temperature *pof1-6* cells also become small, as described previously. Again if this were a normal response to cadmium wild type cells should show a similar decrease in size. Thus samples were taken from the same cultures used for the viability experiment above and the lengths of 100 cells for each timepoint measured. In agreement with our prediction, wild type cells became smaller in response to cadmium as did *pof1*-6 cells even at the permissive temperature (Figure 6.7). Although this size decrease was less drastic than that seen in *pof1-6* mutants at restrictive temperature, the fact that it did not occur in *zip1* cells, which actually increased in size, suggests that it is biologically significant.

Cells exposed to cadmium undergo a wave of septation in a Zip1-dependent manner

Finally whilst observing the cadmium exposed cells I noticed that a larger than average number of them were septating. The *cdc15*$^{+}$ gene, whose product is known to be involved in the positive regulation of septation, appeared to be transcribed in response to cadmium in a Zip1-dependent manner (Tables 5 and 6). Thus I tested whether this septation response was Zip1-dependent. Wild type and *pof1*-6 cells showed a peak of septation 2-4 h after cadmium exposure, whilst the percentage of septated cells in *zip1* mutants did not display such an increase (Figure 6.8 and Chapter 8.2 for details of septation analysis). The biological significance of this remains unclear but it is obviously a Zip1-dependent response.

Summary

This chapter has described attempts made to identify the function of Zip1. Cells lacking functional *zip1*$^{+}$ are specifically sensitive to cadmium stress. Since Zip1 is a bZIP transcription factor it is likely that this is because some transcriptional response normally initiated in response to cadmium is missing in these cells. Microarray analysis has been used to identify a set of 27 genes whose expression is reduced by more than 50% in a *zip1* mutant. All of these genes are induced by cadmium in a wild type cell. 19 of these genes are normally induced more than two-fold in response to cadmium and the majority of these are known to be expressed specifically in response to cadmium stress in wild type cells, as opposed to being expressed in a general response to stress. These data indicate that Zip1 initiates the transcription of a specific set of cadmium response genes when cells are exposed to cadmium. Although several genes of the sulphate assimilation pathway appear to be transcribed in a Zip1-dependent manner, cells defective in Zip1 function can grow on media containing only an inorganic sulphur source, suggesting that Zip1 is not essential for this pathway.

Zip1 is seen to accumulate in a modified form in cadmium-treated cells. This modified form appears to be a similar molecular weight to that seen to accumulate in *pof1-6* mutants at restrictive temperature, suggesting that both cadmium exposed cells and *pof1* mutants have increased Zip1 activity. Wild type cells and *pof1-6* mutants show a growth arrest and cell-size reduction when exposed to cadmium, but remain viable. In contrast cells lacking *zip1* do not show a decrease in cell size and rapidly loose viability following cadmium exposure. Although they do not increase in number, wild type and *pof1-6*

mutants also undergo a wave of septation following cadmium treatment and this appears to be Zip1-dependent.

Discussion

The data presented in this chapter provides strong evidence that Zip1 transcription is part of a cadmium-specific stress response. This is for the following reasons; firstly cells with a nonsense *zip1* allele are specifically sensitive to cadmium stress. Secondly all genes found to be dependent upon Zip1 for expression are induced by cadmium and over half of them are genes which have previously been shown to be part of a cadmium- specific transcriptional response. Thirdly, levels of the Zip1 protein itself increase following exposure to cadmium, suggesting increased Zip1 activity, and finally cells defective in Zip1 function rapidly lose viability when exposed to cadmium stress compared to wild type cells.

It was previously shown that the transcriptional response to cadmium involved, as well as the expression of the CESR, the expression of 32 stress specific genes. Most of the CESR genes are dependent upon the Sty1 pathway for induction, thus *sty1Δ* cells are cadmium sensitive. However many of the cadmium specific genes identified were found to be not regulated by Sty1, thus it was postulated that there were stress specific regulators acting independently of Sty1. (Chen, D. et al., 2003) Zip1 is a likely candidate to be one of these regulators. It is likely that the key role of Zip1 is to induce components of the sulphate assimilation pathway to increase GSH production. GSH can bind to cadmium and is eventually removed from the cell via the vacuole (Penninckx, M., 2000). The cadmium specific genes identified previously contained a select group of the sulphate assimilation pathway genes and all of the members of this group appear to be Zip1-

dependent. Interestingly, a much smaller number of genes in this pathway appear to be induced by cadmium in *S. pombe* compared to the number induced in *S. cerevisiae* (Chen, D. et al., 2003). This implies that Zip1 can upregulate GSH production by increasing the expression of a relatively small number of enzymes. The sulphate transporter, C869.05c, appears to be massively upregulated in response to cadmium, thus it may be that increasing sulphur uptake is the key factor in increasing GSH production. These data suggest that the sulphate assimilation pathways may function differently in the two organisms. As well as the sulphate pathway, Zip1 appears to have evolved to also regulate some other genes whose function in cadmium stress is less obvious. In particular there are 4 genes whose expression increases more than 20-fold in response to cadmium in a Zip1-dependent manner: C965.06, a predicted potassium channel subunit and PB2B2.08, C1348.06c and C977.05c, of unknown function. Potassium is required for many physiological functions in the cell, including pH maintenance, thus it makes sense for the cell to upregulate potassium transport, particularly since cadmium stress involves the exchange of many different ions in the cell (Cd^{2+}, SO_4^-, Zn^{2+}) and is likely to affect intracelluar pH maintenance dramatically. In fact this gene is part of the defined CESR, thus is not surprising that it would have a major role in cadmium stress. However it is interesting that this is expressed in a Zip1-dependent manner, suggesting Zip1 can express CESR genes specifically in response to cadmium. Zip1 is not sensitive to other stresses thus it is likely some other factor expresses this gene in other conditions and Zip1 carries out this task only under cadmium stress. It is obviously difficult to say what the role of the other three highly expressed genes is. C1348.06c and C977.05c, although of unknown function, have homologues which are induced during Zinc limitation. Cadmium is able to deplete zinc from proteins. Thus it is likely that these genes are induced in response to the depletion of zinc caused by cadmium stress. It will be

interesting to see if Zip1 is also required to induce these genes in response to zinc depletion where there is no cadmium stress.

If Zip1 is a cadmium specific transcription factor some mechanism must exist to switch on Zip1 activity when cells are exposed to cadmium. The accumulation of a modified form of Zip1 in cells exposed to cadmium suggests that it is this modification which switches on Zip1 activity. The fact that this modified form is a similar molecular weight to the phosphorylated species observed in *pof1-6* mutants suggests that both bands are the same modified form of Zip1. If Zip1 is phosphorylated in response to cadmium some protein kinase must be responsible. An obvious candidate is Sty1, but since most of the genes downstream of Zip1 appear to be transcribed in response to cadmium independently of Sty1 this seems unlikely. Thus there must be some other cadmium-stress activated kinase able to phosphorylate and activate Zip1. Interestingly several of the cadmium specific genes downstream of Zip1, including Pof1, have been shown to be super-induced in *atf1Δ* strains (Chen, D. et al., 2003). This suggests that Atf1 may act antagonistically to Zip1; this could potentially be through the kinase which phosphorylates Zip1.

Although several genes involved in the sulphate assimilation pathway are transcribed by Zip1, *zip1* mutants are able to grow with only an inorganic sulphur source. This suggests that the pathway is still relatively functional in these mutants. This is in direct contrast to the *S. cerevisiae* homologue of Zip1, Met4, which is essential for this pathway. This is probably because Met4 transcribes far more of the sulphate assimilation genes than Zip1 and suggests that in *S. pombe* some other transcription factor is involved in the transcription of these genes in addition to Zip1. It is likely that Zip1 is only important for maximising the output of this pathway in times of stress, when lots of product is needed. In stress-free conditions some other factor is

probably sufficient for transcription. Why *S. cerevisiae* came to rely on Met4 for regulation of sulphur assimilation in normal growing conditions whilst *S. pombe* only uses Zip1 when exposed to cadmium stress remains unclear. Possibly budding yeast originally grew in conditions where they were almost constantly exposed to cadmium, thus required a constantly high production of sulphur compounds and eventually evolved to rely on Met4 completely for this pathway, whereas fission yeast were less exposed to cadmium thus generally didn't require Zip1 activity and could develop a separate pathway for non-stressed growing conditions.

The data regarding the physiological response of fission yeast to cadmium suggests that the phenotype seen in *pof1* mutants at restrictive temperature is the consequence of an inappropriate cadmium response. If the primary role of Zip1 is to transcribe genes in response to cadmium stress and *pof1* mutants have too much Zip1 activity then this appears to make sense, the *pof1-6* cells arrest growth and get smaller at restrictive temperature because they are acting like cells exposed to cadmium. However, there are two problems arising from this idea. Firstly, if phosphorylation is required for the transcriptional activity of Zip1 in response to cadmium, yet this phosphorylation occurs in *pof1* mutants, where there is no cadmium, how is this phosphorylation regulated? The fact that the phosphorylation can occur in *pof1-6* mutants when there is no cadmium suggests either that it occurs by default when Zip1 levels increase, or Pof1 itself normally negatively regulates the kinase responsible for the phosphorylation. If either of these mechanisms are true, inhibition of Pof1 must be involved in the switching on of Zip1 activity, either purely by increasing Zip1 levels, or by increasing Zip1 levels and at the same time switching on the kinase required for Zip1 activation. Thus there must exist some mechanism for cadmium to switch Pof1 activity off.

The second problem to arise from the idea that the growth arrest seen in *pof1* mutants is a result of an inappropriate cadmium response is the question of how the cells normally arrest growth yet remain viable in response to cadmium. This must occur because of the activation of some gene (or genes) downstream of Zip1. The sulphate assimilation pathway genes are the major Zip1 targets thus it is possible that this pathway has some role in the growth arrest seen in cadmium-exposed cells. As illustrated in Figure 6.9, in addition to sulphur amino acids and GSH, the sulphate assimilation pathway is used to produce S-AdenosylMethionine (AdoMet). AdoMet can be used as a methyl-group donor and thus can be involved in many different reactions, however it can also be decarboxylated and its amino-propyl group used for polyamine synthesis (Thomas, D. and Surdin-Kerjan, Y., 1997). Polyamines are absolutely required for eukaryotic and prokaryotic cell growth (Tabor, C.W. and Tabor, H., 1984) although very few of the molecular functions of polyamines *in vivo* are known. It is believed that the production of the amino acid hypusine from the polyamine spermidine represents one of the critical functions of polyamines, since hypusine is required for the production of the elongation initiation factor eIF5A, a protein synthesis initiation factor which is essential for growth (Chen, K.Y. and Liu, A.Y., 1997; Park, M.H. et al., 1997; Park, M.H. et al., 1993; Schnier, J. et al., 1991). Intriguingly, recent studies of the requirement of polyamines for the growth of fission yeast revealed that fission yeast cells unable to synthesise spermidine arrested growth as small cells with vesicle-like bodies and a minor accumulation of cells in G1-phase (Chattopadhyay, M.K. et al., 2002), a phenotype which has not been previously observed in *S. pombe* and is remarkably similar to *pof1* mutants at restrictive temperature. Even more intriguingly, data from cadmium stress transcriptional profiles shows that while many of the genes of the sulfate assimilation pathway are upregulated in response to cadmium stress, many of the genes leading from AdoMet to spermidine production are in fact repressed by cadmium (Figure 6.9 and (Chen, D. et al., 2003). It is thus

possible that Zip1 acts to upregulate the sulphate assimilation pathway which leads to increased GSH production, thus conferring cadmium resistance and at the same time downregulates the production of spermidine, either directly through the genes required for spermidine production or simply by making so much GSH that there are less sulphur compounds available to make polyamines. This would lead to cell cycle arrest until cadmium was removed, GSH production dropped and spermidine levels returned to normal. Although this is purely hypothetical, polyamines are already known to be involved in stress responses in plants, although they tend to increase in these conditions (Richards, F.J. and Coleman, R.G., 1952; Slocum, R.D. et al., 1984). It is however conceivable that since both spermidine and GSH rely upon the same sulphur compounds for their production fission yeast evolved to use a decrease in spermidine levels as a growth arrest mechanism specifically in response to cadmium stress.

Alternatively the growth arrest mechanism may be due to some other, non-sulfur assimilation pathway gene downstream of Zip1. As discussed in Chapter 1, protein translation is believed to be a key mechanism for the regulation of cell growth. It is conceivable that some single gene downstream of Zip1 is able to act to arrest growth, for instance through inhibition of protein translation. The three genes of unknown function which are highly expressed in response to cadmium in a Zip1-dependent manner, PB2B2.08, C1348.06c and C977.05c, are potential candidates for this type of function.

The final enigma to arise from the data presented in this chapter concerns the septation response seen to occur in the cadmium-treated cells. This is a very clear response, with almost 40% of wild type cells septating following cadmium exposure. It is very strange that cells would wish to undergo division when exposed to such toxic conditions. One possible explanation is that this is a mechanism used to reduce cell size. Cell starved of nitrogen have been

shown to undergo stimulated rates of division, which results in a larger number of cells but at a reduced size (Young, P.G. and Fantes, P.A., 1987). Cells exposed to cadmium undergo some growth arrest mechanism which is similar to if they are starved, thus possibly this reduced size is necessary to withstand the growth arrest. The fact that no increase is seen in cell number following septation (compare Figures 6.6 and 6.8) suggests that although the cells divide they do not increase significantly in size, thus are not detected by the cell counter. It appears that the septation response is Zip1-dependent, thus Zip1 may be the factor which couples a cell size reduction with cell growth arrest.

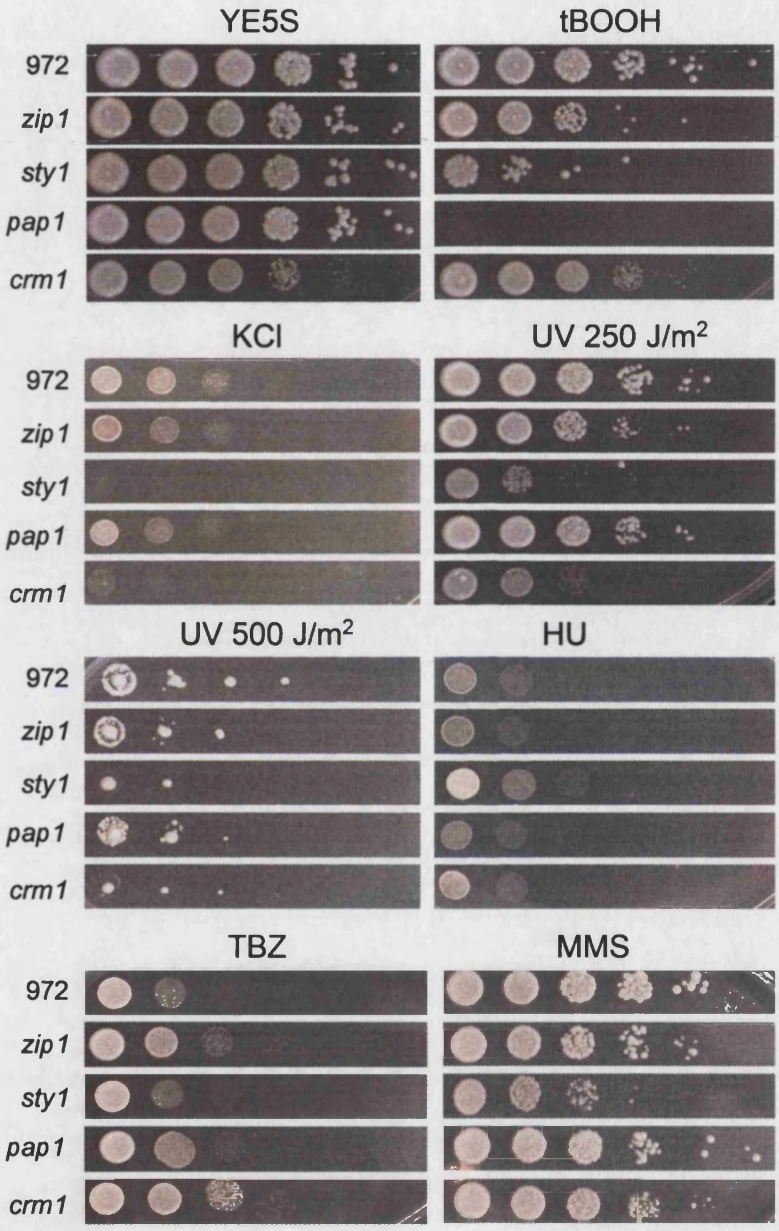


Figure 6.1 Testing the sensitivity of *zip1* to different stresses.

Wild type (top row), *zip1* (second), *sty1* (third), *pap1* (fourth)-deleted cells or *crm1-809* cells (bottom) were spotted onto rich plates in the absence (top left) or presence of 0.5mM tBOOH, 1M KCl, 10mM HU, 20μg/ml TBZ or 0.02% MMS. Alternatively cells were spotted onto rich plates then exposed to 250 or 500 J/m^2 UV irradiation.(10^6 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightwards) Plates then incubated at 26°C 4 d.

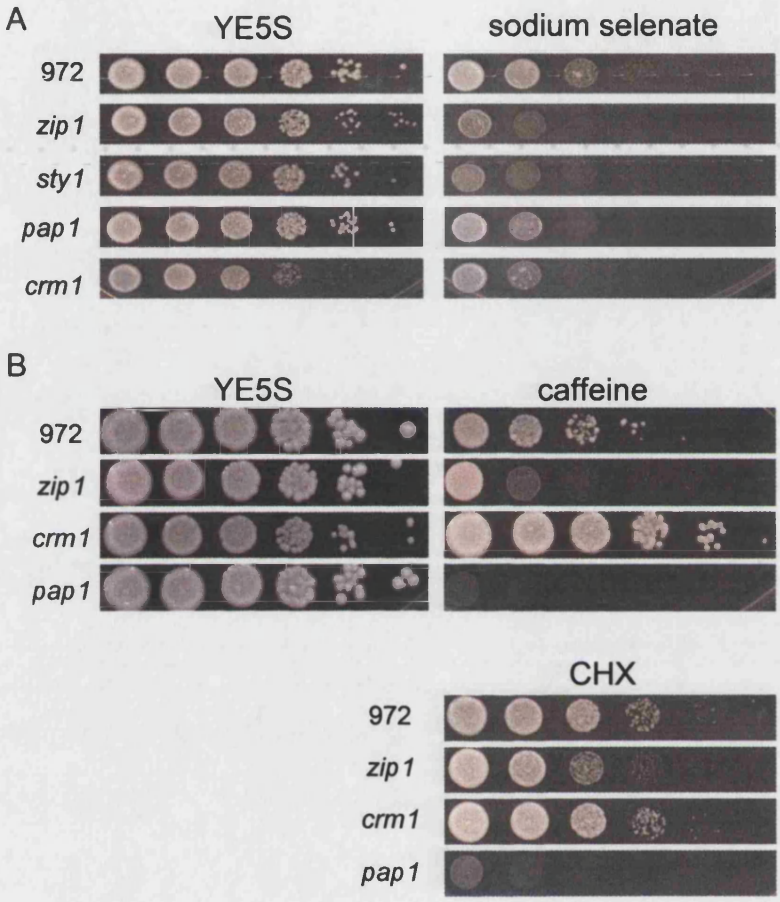


Figure 6.2 Testing the drug sensitivity of *zip1*.
Wild type (top row), *zip1* (second), *crm1-809* (third) or *pap1*-deleted cells (bottom) *or* were spotted onto rich plates in the absence (left) or presence of **A** 50μM sodium selenate or **B** 12.5mM caffeine or 15μg cycloheximide.(10^6 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightwards) Plates then incubated at 26°C 4 d.

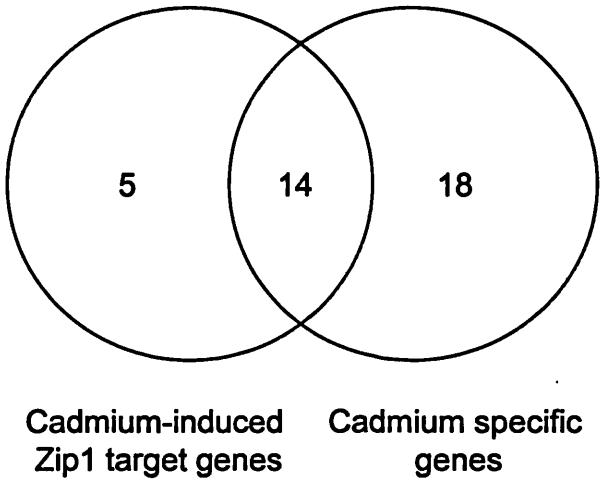


Figure 6.3 Comparison of Zip1-target and cadmium-specific genes.
Summary of DNA microarray analysis. Genes induced by cadmium stress in a Zip1-dependent manner and those induced in a cadmium stress-specific manner (Chen, Toone et al. 2003) are compared.

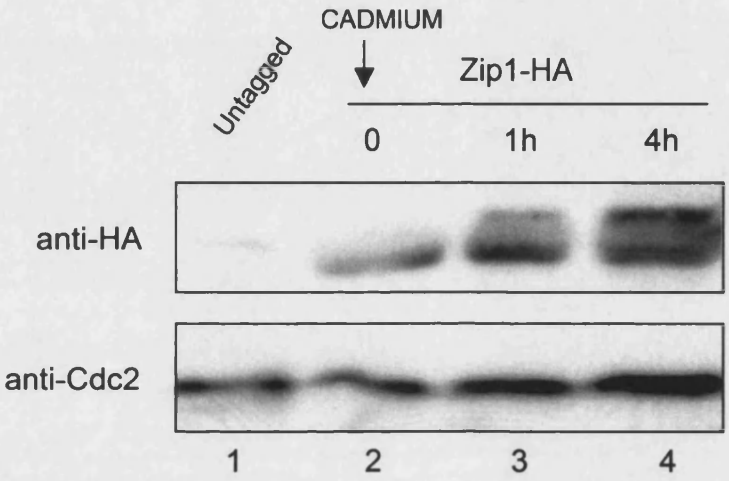


Figure 6.4 Accumulation of Zip1 upon cadmium exposure.

Increase of Zip1 upon cadmium exposure. Exponentially growing wild type cells containing Zip1-HA were treated with 0.5 mM cadmium sulphate and protein extracts were prepared at 0, 1 and 4 h time points (lanes 2 to 4). Immunoblotting was performed with anti-HA (upper) or anti-Cdc2 antibody (lower). Untagged wild type was used as a negative control (lane 1).

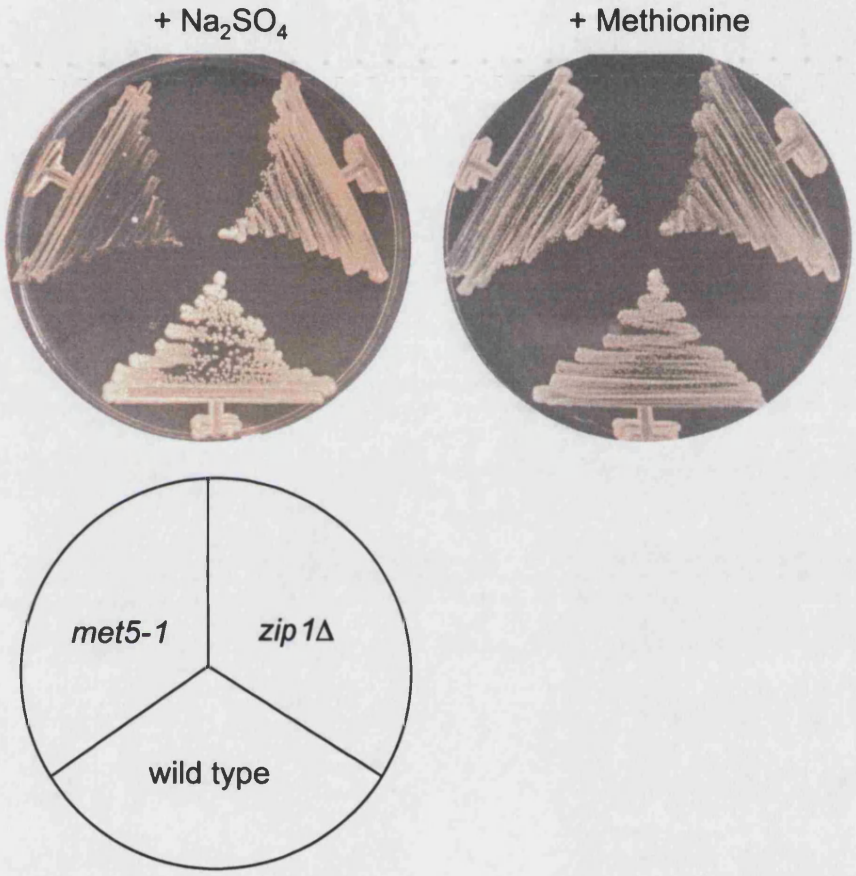


Figure 6.5 The absence of Zip1 does not result in sulphur amino acid auxotrophy.
Wild type, *zip1*$^+$-deleted and *met5-1* mutant cells were streaked on minimal medium whose sulphur sources are provided from only Na_2SO_4 (left) or methionine (right) and incubated at 26°C for 5 d. Some growth of *met5-1* in the presence of Na_2SO_4 is most likely ascribable to the existence of residual methionine pool inside *met5-1* cells.

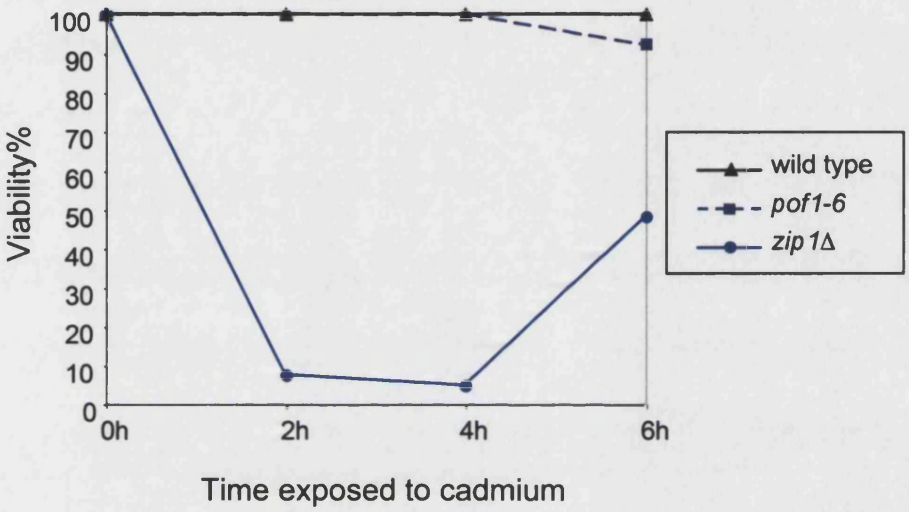


Figure 6.6 Zip1 is required for maintenance of viability during cadmium exposure.

Viability under cadmium stress. Wild type (triangles), *pof1-6* (squares) or *zip1*-deleted cells (circles) were treated with 0.5 mM cadmium sulphate at 26°C and cell viability was examined every two hours interval. At each time point, 2x 200 cells were plated onto rich medium (lacking cadmium) and incubated at 26°C for 5 d. The number of viable colonies was counted and viability was calculated by dividing this number by 200 and calculating an average.

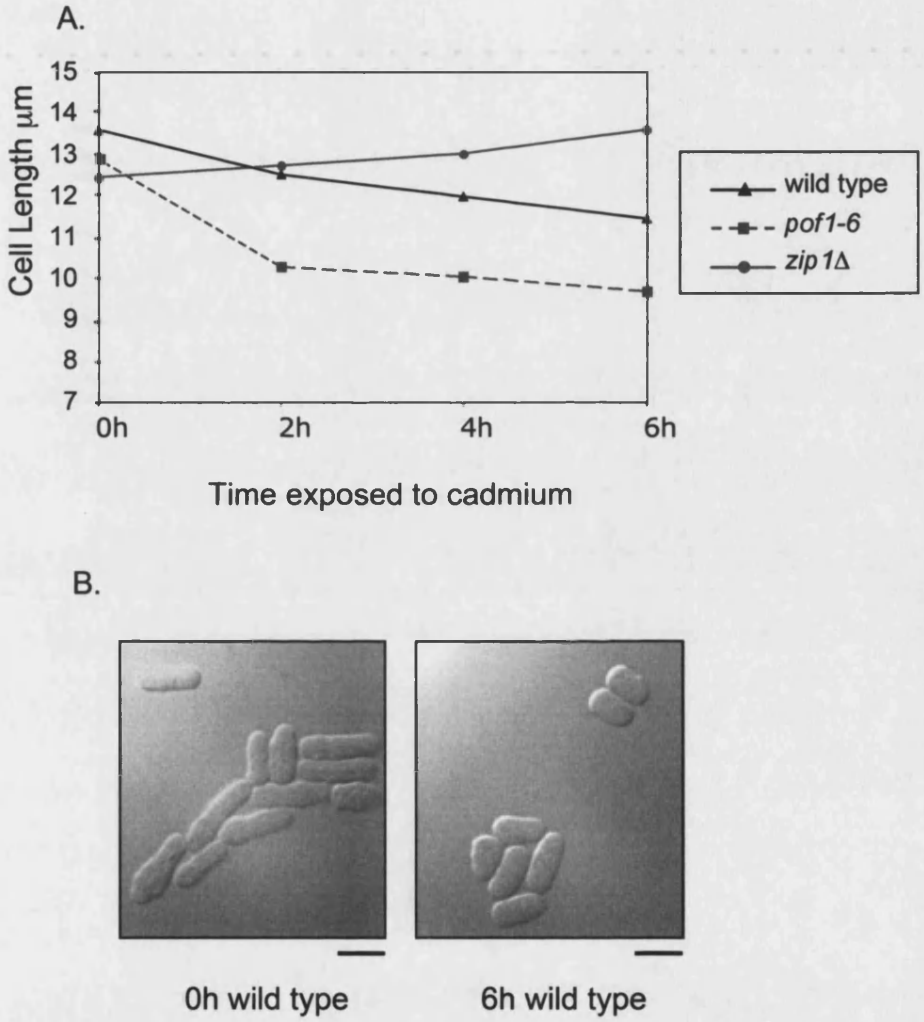


Figure 6.7 Cadmium induces a Zip1-dependent cell size decrease.

Cell size change upon cadmium exposure.(A) Wild type (triangles), *pof1-6* (squares) or *zip1*-deleted cells (circles) were treated with 0.5 mM cadmium sulphate at 26°C and the cell size was measured and plotted (n=100 per time point) every 2h. (B) Cell morphology under cadmium stress. Phase contrast micrographs of wild type cells before (left) or after cadmium treatment (6 h, right) are shown. The bar indicates 10 µm.

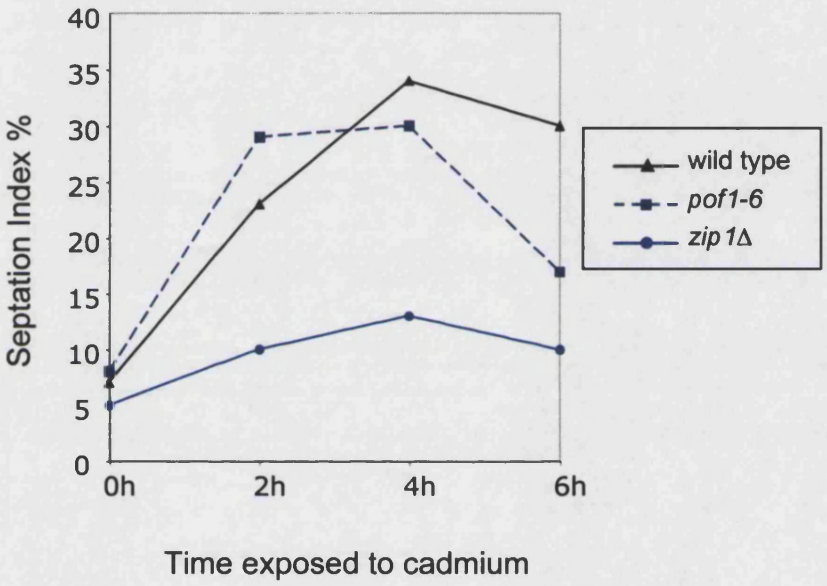


Figure 6.8 Cadmium Induces Zip1-dependent septation.
Wild type (triangles), *pof1-6* (squares) or *zip1*-deleted cells (circles) were treated with 0.5 mM cadmium sulphate at 26°C and the number of septating cells was counted every 2h and plotted (n=100 per time point)

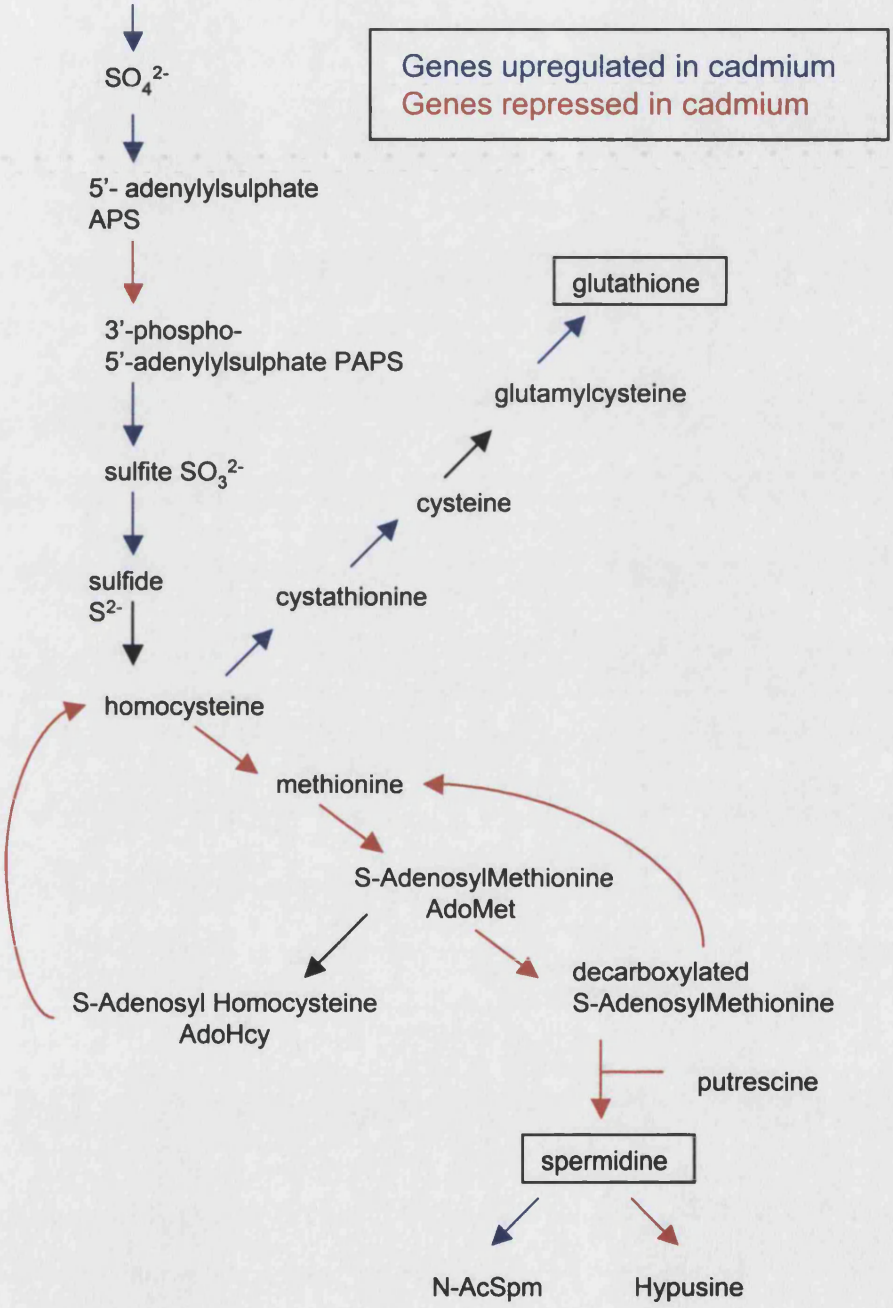


Figure 6.9 The downstream products of the sulphate assimilation pathway.

Arrows drawn in blue represent process where the genes responsible are upregulated in response to cadmium, arrows drawn in red represent processes where the genes responsible are downregulated in response to cadmium and black arrows are where data is not known (Chen, Toone et al. 2003). Note that most of the processes leading to glutathione production are upregulated whereas most leading to the production of the polyamine spermidine are downregulated.

Table 5. Zip1 target genes

Gene name	Function.
C869.05c	**Sulfate transporter, similar to S.cerevisiae Sul1**
C1739.06c	**Uroporphyrin methyltransferase**
PB1C11.03	**Predicted membrane transporter**
PB10D8.02c	**Predicted arylsulfatase**
PB10D8.01	**Predicted membrane transporter**
pof1	**F-box protein**
C106.17c	**Predicted homoserine O-acetyltransferase, similar to *S. cerevisiae* Met6**
PB2B2.08	**Unknown function.**
P16F5.08c	**Predicted flavin dependent monooxygenase**
C10F6.01c	Predicted Sulfite reductase, similar to *S. cerevisiae* Ecm17
frp1	Ferric-chelate reductase
P8B7.05c	Predicted carbonic anhydrase
cad1/ hmt2	**Sulfide-quinone oxidoreductase**
C725.04	**oxalyl-CoA decarboxylase**
C23A1.14c	**Prdicted cystathionine gamma-synthase**
C584.01c	Predicted sulfite reductase, similar to *S. cerevisiae* Met10
C965.06	**Predicted potassium channel subunit**
PB10D8.04c	Predicted malate permease
C1827.03c	Predicted long-chain fatty acid transporter
C27.04	**Coiled-coil protein, homology to *S. cerevisiae* Rad50**
fip1	Iron permease
C1348.06c	**Unknown function. Has strong similarity to SPBC977.05c and SPBPB2B2.15**
C977.05c	**Unknown function. Has strong similarity to SPBC1348.06c and SPBPB2B2.15**
cdc15	Involved in cytokinesis and septation.
PB2B2.05	**Predicted GMP synthase**
cdc22	**Ribonucleoside-diphosphate reductase (large subunit)**
C622.12c	**Predicted NADP-specific glutamate dehydrogenase**

Table 6 Expression levels of Zip1 target genes in wild type and *zip1* mutant cells exposed to cadmium stress compared to basal wild type level.

Gene	Expression levels (normalized to 0 minutes wild type)					
	wild type			*zip1*		
	0	15	60	0	15	60
C869.05c	1	16.44554	11.52475	0.399	0.623762	0.861386
C1739.06c	1	7.760504	3.760504	0.44	0.495798	0.617647
PB1C11.03	1	4.141304	1.691304	1.156	0.45	
PB10D8.02c	1	7.325153	11.53374	0.407	0.840491	
PB10D8.01	1	2.708428	2.596811	0.477	0.373576	0.874715
C106.17c	1	3.532408	2.217593	0.53	0.513889	
PB2B2.08	1	7.817204	25.65591		1.354839	2.11828
P16F5.08c	1	2.978022	4.398352	1.072	0.664835	0.491758
C10F6.01c	1	1.275081	1.088457	0.617	0.374326	0.272923
frp1	1	3.808785	2.728682	0.819	1.341085	0.718346
P8B7.05c	1	1.664567	1.662992	0.588	0.625197	0.533858
cad1/ hmt2	1	2.245763	1.79661	0.586	0.855932	0.794915
C725.04	1	2.962306	2.760532	1.039	1.190687	
C23A1.14c	1	5.793706	3.531468	1.03	2.391608	
C584.01c	1	1.307344	0.728014	0.923	0.584769	0.363554
C965.06	1	6.070707	21.77778	1.174	2.757576	11.78788
PB10D8.04c	1	1.195674	0.344149	0.503	0.556539	0.235005
C1827.03c	1	1.175439	1.176535	0.863	0.574561	0.371711
C27.04	1	0.941022	2.580603	1.365	0.985583	0.584535
fip1	1	0.522767	1.288967	0.86	0.367776	0.318739
C1348.06c	1	2.224299	21.04673		1.448598	6.009346
C977.05c	1	1.846847	20.28829		1.009009	6.162162
cdc15	1	0.572368	1.79057	0.951	0.589912	0.548246
PB2B2.05	1	2.288754	5.571429		1.589666	2.270517
cdc22	1	0.855901	2.209938	1.169	0.685714	0.96646
C622.12c	1	1.288372	2.617054	1.237	1.756589	1.296124
pof1	1	5.726501	2.091169		1.107713	1.63327

Table 7 The dependency of Cadmium specific, Zip1-dependent genes on Sty1/Atf1 in a wild type cell (Sty1/Atf1 data taken from Chen, Toone et al. 2003)

Gene name	Function.	Sty1/Atf1 dependent?
C869.05c	**Sulfate transporter, similar to S. cerevisiae Sul1**	**No**
C1739.06c	**Uroporphyrin methyltransferase**	**No**
PB10D8.02c	**Predicted arylsulphatase**	**Sty1/Atf1**
PB10D8.01	**Predicted membrane transporter**	**Atf1**
pof1	**F-box protein**	**No**
C106.17c	**Predicted homoserine O-acetyltransferase, similar to *S. cerevisiae* Met6**	**No**
PB2B2.08	**Unknown function.**	**No**
P16F5.08c	**Predicted flavin dependent monooxygenase**	**No**
cad1: hmt2	**Sulfide-quinone oxidoreductase**	**Unknown**
C725.04	**oxalyl-CoA decarboxylase**	**Unknown**
C23A1.14c	**Predicted cystathionine gamma-synthase**	**No**
C1348.06c	**Unknown function.**	**No**
C977.05c	**Unknown function.**	**No**
PB2B2.05	**Predicted GMP synthase**	**No**

Chapter 7

Discussion

Introduction

This thesis has described the characterisation of the fission yeast F-box protein Pof1. Since Pof1 is one of only two F-box proteins in this organism which are completely essential to cell growth, discovering the substrate of SCF^{Pof1} will give an important insight into why the SCF complex is essential *per se* in fission yeast. In order to identify the substrate of Pof1, temperature sensitive *pof1* mutants were generated and a suppressor mutation, able to rescue the growth of these mutants at restrictive temperature, was isolated. This was found to be a nonsense allele of the *zip1* gene. Zip1 is a bZip1 transcription factor. The primary defect in cells lacking Zip1 function is sensitivity to the heavy metal cadmium. Microarray analysis of changes in gene expression in response to cadmium in a wild type and *zip1* strain suggested that the primary cause of this cadmium sensitivity was that Zip1 is responsible for the upregulation of the sulphate assimilation pathway genes, which is needed for an increase in glutathione production in response to cadmium. The phenotype seen in *pof1* mutants at restrictive temperature and wild type cells overexpressing Zip1 is growth arrest and small cell size. Since Zip1 seems to have a primary role in cadmium stress response, this is likely to be a response normally required for cadmium stress resistance. This idea is further supported by the fact that cells exposed to cadmium show a similar phenotype which is Zip1-dependent.

This Chapter will attempt to bring together the data presented in this thesis into a final model explaining the function of Pof1 and Zip1 and the mechanisms which exist to control the activity of each protein and their relationship to each other.

7.1 A model for the function and regulation of Zip1

A model of how Zip1 may be regulated through the activity of SCF^{Pof1} is shown in Figure 7.1. All of the data suggests that high levels of Zip1 activity are required during times of cadmium stress. However, in normally growing, unstressed cells, Zip1 is at a relatively low level and has a short, Pof1-dependent halflife. This suggests that the SCF^{Pof1} is constantly ubiquitylating Zip1 in these conditions, leading to its degradation. A cell in normal conditions probably has very little Zip1 transcriptional activity. When cells are exposed to cadmium Zip1 activity must increase. Cells exposed to cadmium show an increase in Zip1 levels, mostly through the accumulation of a slow-migrating, modified form of Zip1, which most probably represents phosphorylation. Since it accumulates in cadmium this phosphorylated form is likely to represent a transcriptionally active form of Zip1. As discussed previously, since this form accumulates in a *pof1* mutant in the absence of cadmium, switching off Pof1 activity must be sufficient for its accumulation. Thus the response of the cell to cadmium is likely to involve the switching off of SCF^{Pof1} activity (or some upstream kinase; see below) to allow accumulation of Zip1. The role of Pof1 in the cell is thus to keep Zip1 activity low under normal growing conditions, but high in response to cadmium stress.

7.2 Regulation of Zip1 activity through phosphorylation

As discussed in Chapter One many SCF substrates are known to be regulated by phosphorylation. Zip1 appears to be regulated in the same way. Two modified forms of Zip1 are observed in a *pof1* mutant, both of which are phosphorylated. The faster migrating of these two forms interacts with Pof1 and is stabilised in a *pof1* mutant. This suggests that this form carries the phosphorylation signal which normally leads to interaction with Pof1 and subsequent degradation. The kinase responsible for this phosphorylation (shown as 'kinase 1' in Figure 7.1) is likely to be constitutively active, since Zip1 appears to be unstable in normal growing conditions. A prime candidate for this kinase was casein kinase II (Hathaway, G.M. and Traugh, J.A., 1982), a constitutively active kinase which has been shown in fission yeast to be required for polarized growth, although not the establishment of polarity (Snell, V. and Nurse, P., 1994). However casein kinase II mutants showed no change in Zip1 stability (data not shown) thus the kinase responsible for Zip1 phosphorylation and subsequent degradation remains unidentified. This kinase would also be a good target for the cadmium stress response, since switching off this phosphorylation would lead to increased Zip1 levels. This would also explain how Pof1 can be upregulated by Zip1 under cadmium stress (Chapter 6) without leading to an immediate degradation of Zip1, Pof1 levels could increase to ensure they were sufficient for future Zip1 degradation but would not start to degrade Zip1 until it was phosphorylated.

As discussed above the other phosphorylated form of Zip1 appears to be required for the cadmium-stress response. The kinase responsible for this phosphorylation (shown as 'kinase 2' in Figure 7.1) has also not been identified. The fact that this phosphorylation occurs in *pof1* mutants suggests either it is constitutive once Zip1 starts to accumulate or the kinase responsible is negatively regulated by Pof1. Why the cell requires this further round of phosphorylation is puzzling. It is possible that it is required for full activation of Zip1 activity. If this is true it appears dangerous for the cell to

regulate Zip1 turnover and activation through the same complex, SCF^{Pof1}, since, as seen in *pof1* mutants, inactivation of this complex is completely toxic to the cell. If the phosphorylation were regulated by a different mechanism to the degradation it would provide more of a safety net to the cell if Pof1 were to malfunction. The fact that the cell uses Pof1 to completely control Zip1 activity suggests that to the evolving fission yeast cell cadmium exposure was a far greater risk to survival than unregulated Zip1 activity.

7.3 Pof1 and Zip1 function differently to Met30 and Met4

The roles of Pof1 and Zip1 seem different to those of their budding yeast homologues Met30 and Met4. Met4 is required for growth in conditions where there is no exogenous supply of sulphur amino acids. Zip1 however is not essential in these conditions. The differences in function between the two is probably the reason for the different modes of their regulation; there is no evidence of degradation-independent regulation of Zip1 by ubiquitylation, in direct contrast to Met4 which appears to be ubiquitylated but not degraded in normal growing conditions. This is likely to be because budding yeast cells require a constant Met4 activity whereas fission yeast do not so can simply degrade Zip1 when not under cadmium stress. Met4 ubiquitylation has been shown to alter its affinity for different promoters (Kuras, L. et al., 2002). It is possible that ubiquitylation prevents the full transcriptional activity of Met4 required for a cadmium-stress response but allows it still to be used for survival in low methionine conditions.

7.4 Cadmium-stress in higher organisms

Cadmium is one of the most serious environmental pollutants. It has a biological half-life of 10-30 years (Hengstler, J.G. et al., 2003), thus accumulates in the body and has been shown to be mutagenic through its ability to inactivate mismatch repair mechanisms (Jin, Y.H. et al., 2003). Given that many cells in the body will be exposed to cadmium it is likely that higher organisms have conserved some aspects of cadmium-stress response seen in lower eukaryotes. Mammalian cells have been shown to induce many genes involved in oxidative stress response following exposure to cadmium. The bZIP transcription factor NF-E2-related factor 2 (Nrf2) has been shown to be an important transcription factor for the upregulation of these genes (Alam, J. et al., 1999; Huang, H.C. et al., 2000; Itoh, K. et al., 1997; Moinova, H.R. and Mulcahy, R.T., 1999; Nguyen, T. et al., 2000; Venugopal, R. and Jaiswal, A.K., 1996; Wild, A.C. et al., 1999), in particular the overexpression of Nrf2 was shown to lead to upregulation of genes involved in glutathione production in certain cell types (Shih, A.Y. et al., 2003). Interestingly Nrf2 was recently shown to be an extremely labile protein, specifically degraded by the ubiquitin-proteasome system. However in the presence of cadmium Nrf2 became stabilised and was thus transcriptionally active (Stewart, D. et al., 2003). This is an identical mechanism to that which seems to exist to control Zip1 activity, suggesting that Zip1 and Nrf2 are functional homologues and that the degradation of Nrf2 may be SCF dependent. Thus the mechanism used by fission yeast to upregulate glutathione production in response to cadmium is potentially conserved in higher organisms. However no reports exist of Nrf2 being able to activate a growth arrest mechanism, similar to that seen in high levels of Zip1 activity. Since in multicellular organisms cells must co-operate with neighbouring cells it is possible that the growth arrest in response to cadmium is regulated by a different system to that which regulates glutathione production, which is likely to take into account signals from the surrounding cells. It is also likely that mammalian cells have alternative responses to cadmium stress, such as apoptosis, which are not

necessary in fission yeast. Thus although both increased production of glutathione and arrested growth are likely to be conserved responses to cadmium in all organisms, it is likely that only lower organisms couple both to the same transcriptional activity. It remains to be seen whether mammalian transcription factors responsible for modulation of growth in response to cadmium are regulated by SCF complexes.

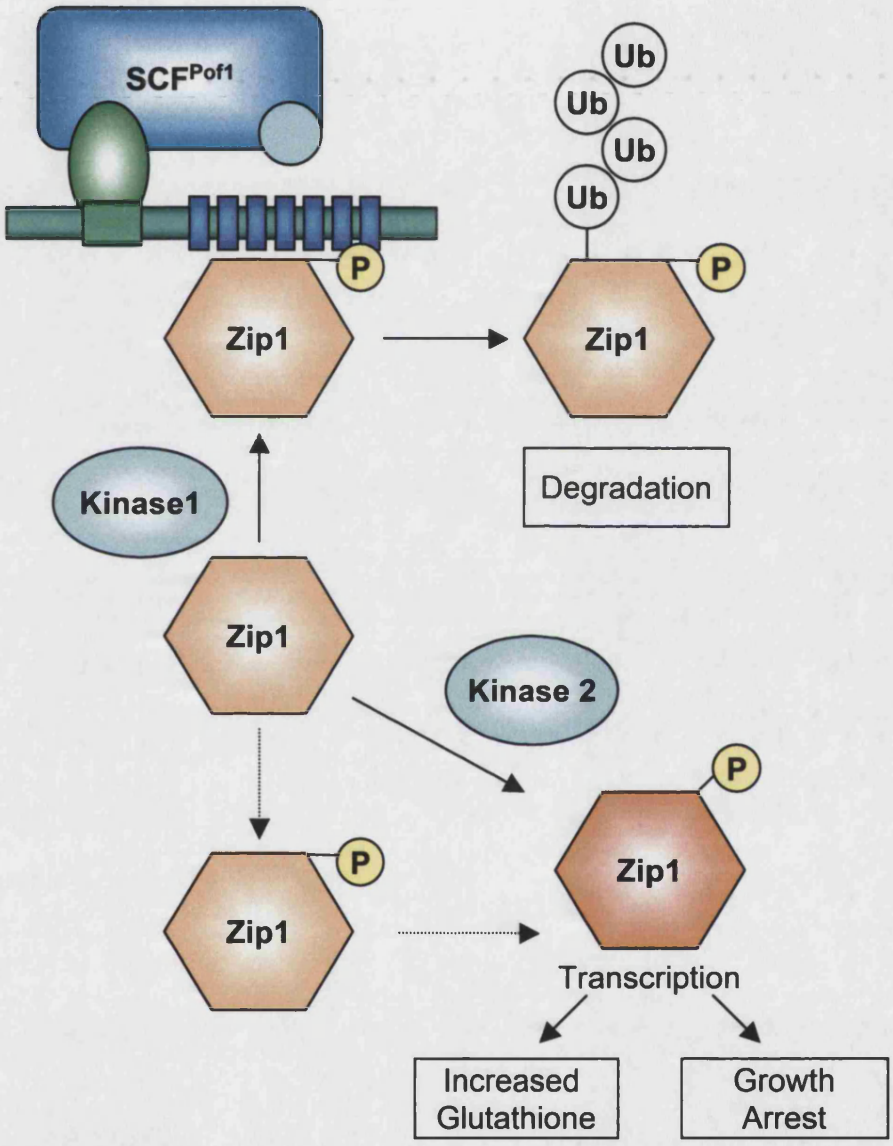


Figure 7.1. A model for the function and regulation of Zip1.
Zip1 is constitutively produced, but its cellular levels are kept low under normal, non-cadmium, growing conditions (top).This is achieved by constitutive SCF^{Pof1} dependent degradation. Under cadmium stress, some switch triggers an increase in Zip1 protein levels and its transcriptional activity. This results in an increased production of glutathione coupled with an arrest of cell growth, thereby ensuring high cell viability during cadmium exposure. The mechanism of this switch is unknown but the accumulation of a phosphorylated Zip1 species (emphasized with dark orange) during cadmium exposure suggests that the phosphorylated form could play some role in Zip1 transcriptional activity.

Chapter 8

Materials and Methods

8.1 Laboratory stocks and solutions

All media, pipette tips, glassware and commonly used solutions were provided by Cancer Research UK Central Services

Solutions, buffers and media

The recipes of all solutions, buffers and media used for the experiments described in this thesis are listed below:

DNA Manipulation

TE	10mM Tris-HCl, pH 7.0, 0.1M EDTA
10x loading buffer	60% w/v sucrose, 0.1% bromophenol blue
TBE	45mM Tris base, 1mM EDTA
TAE	40mM Tris-acetate 1mM EDTA
Plasmid solution1 (P1)	50mM glucose, 25mM Tris, pH8.0, 10mM EDTA
Plasmid Solution 2 (P2)	200mM sodium hydroxide, 15 w/v SDS
Plasmid solution 3 (P3)	3M potassium acetate.

SZB buffer	1M sorbitol, 0.1M sodium citrate, pH 7.0, 60mM EDTA, pH 8.0, 0.8% w/v β-mercaptoethanol, 600μg/ml zymolyase
Taq buffer (10x)	200mM Tris-HCl, pH 6.8, 100mM KCl, 30mM $MgSO_4$
Vent buffer (10x)	100mM KCl, 100mM $(NH_4)_2SO_4$, 200mM Tris-HCl (pH8.8), 20mM $MgSO_4$, 1% Triton X-100.

Protein biochemistry

STOP buffer	150mM NaCl, 50mM NaF, 10mM EDTA, 0.006% v/v NaN_3 pH 8.0
HB buffer	25mM MOPS pH 7.2, 60mM β- glycerophosphate, 15mM *p*-nitrophenyl-phosphate, 15mM $MgCl_2$, 15mM EGTA, 1mM dithiothreitol, 0.1mM sodium vanadate, 1% Trition X-100, 1mM PMSF, 20μg/ml leupeptin, 40μg/ml apoprotinin.
RIPA buffer	50mM Tris HCl pH7.5, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS.
5x SDS-PAGE loading buffer	60mM Tris HCl ph6.8, 25% glycerol, 2% SDS, 14.4mM 2-mercaptoethanol, 1% bromophenol blue
SDS-PAGE buffer	25mM Tris, 250mM glycine pH8.3, 0.1% SDS
Transfer buffer	39mM glycine, 48mM Tris base, 20% ethanol

λ-phosphatase buffer	50 mM Tris-HCl pH7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01 % Brij 35, 2 mM $MnCl_2$
Buffer U	8M urea, 0.1M NaH_2PO_4, 50mM Tris-HCl, pH8.0

Media

L-Broth (LB)	170mM NaCl, 0.5% w/v yeast extract, 1% w/v bactotryptone
YE5S	0.5% Difco yeast extract, 3% dextrose, 250mg/ml uracil, adenine, histidine, lysine, leucine.
Minimal medium (EMM)	14.7 mM potassium hydrogen phthalate, 15mM Na_2HPO_4, 93.5mM NH_4Cl, 2% w/v glucose, salt stock, vitamin stock, mineral stock
EMM- NH_4Cl	As above but replace NH_4Cl with 10mM Na glutamate.
EMM – sulphur free	As above but omit Na_2SO_4 from salt stocks and replace with NaCl
PBSA	170mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4

8.2 Yeast physiology

Nomenclature

Fission yeast gene names consist of three italicised letters followed by a number, for example, *pof1*. Mutant alleles of a gene are referred to with the allele number following the gene, for example, *pof1-6*. A wildtype allele will be referred to with a superscript plus-sign, for example, $pof1^+$. When a gene is deleted with a specific marker gene this will be written as the gene name

followed by the gene which it has been deleted with, seperated by two colons, for example, *pof1*::*ura4*$^+$. A gene name followed by the Greek letter delta also means that gene has been deleted, for example *pof1*Δ. Gene products are given the same name as the gene but this is written in non-italics with the first letter in upper case, for example, Pof1. Proteins that have been tagged are written with the protein name followed by the tag, for example Pof1-GFP. Strains carrying the kanamycin resistance gene or not are written as *kan*^{R} or *kan*^{S} respectively. Strains able to grow in the absence of a particular supplement are written as being, for example, leu$^+$, corresponding to the first three letters of the supplement followed by a superscript plus-sign.

Budding yeast gene names consist of three italicised letters in upper case and a number, for example *MET30*. Recessive mutant alleles are written in lower case and italicised, for example *met30*. Gene products are written in non-italics, with the first letter in upper case, for example Met30

Mammalian and *X. laevis* proteins are written as non-italics, with the first letter in upper case, for example Rad18. *C. elegans* proteins are written all upper case, for example FOG-2.

Strain growth and maintenance

Standard techniques were used as described (Moreno, S. et al., 1991b) for growth and maintenance of strains. Strains were grown in either liquid media or on plates, using recipes given in Section 8.1, but adding 1.6% agar for plates. Strains were stored in YE5S with 15% glycerol added, at –70°C.

Transformation of fission yeast

Plasmid transformations were carried out using lithium acetate and PEG as described previously (Ito, H. et al., 1983). Approximately 0.5μg of plasmid DNA

was used for normal transformations and 2mg for library transformations. Strains containing a mutation in the *ura4*$^+$ or *leu1*$^+$ gene were transformed with pREP2 or pREP1 vectors (Basi, G. et al., 1993; Maundrell, K., 1993) which carry the *ura4*$^+$ or LEU2 markers respectively. The *kan*^{R} gene was also used as a selectable marker. For the integration of linear DNA fragments, 10μg of DNA was used to transform cells using the lithium acetate and DMSO method described previously (Keeney, J.B. and Boeke, J.D., 1994).

Production of synchronous cultures

Centrifugal elutriation was carried out using a Beckman J6 centrifuge and elutriator rotor as described previously (Moreno, S. et al., 1991b). Elutriations were performed in YE5S media at 26°C and for the study of temperature sensitivity, cultures were filtered and placed straight into pre-warmed media at 36°C. Synchrony was determined by counting cell number and septation index.

For nitrogen starvation and release experiments cultures of prototrophic strains were grown until exponential phase in YE5S media at 26°C, then filtered, washed and placed into nitrogen-free EMM at 26°C for 16 hours. Cells were then filtered, washed and placed back into YE5S, which in the case of the temperature sensitive cultures had been pre-warmed to 26°C or 36°C. Synchrony was determined using flow cytometry (FACs).

Flow cytometric analysis

Approximately 10^7 cells were fixed in 70% ethanol. After pelleting they were washed in 3ml 50mM sodium citrate, resuspended in 1ml 50mM sodium citrate, 0.1mg RNAse, 2μg/ml propidium iodide and incubated at 26°C overnight. They were then sonicated for 45 seconds (setting 6 on a Soniprep 150 sonicator, MSE) and flow cytometry performed as described previously using a Becton Dickinson FACs Scan, with an excitation wavelength of 488nm and a detection

wavelength of 510-550 nm. The CELL QUEST software was used for data analysis.

Determination of fission yeast cell number and viability

200μl samples of cultures were diluted in 10ml isoton (Beckman Coulter). After sonication cells were counted on a Sysmex Microcell Counter F-800 using the white blood cell channel. Viability was measured by counting cells, plating out 200 cells twice onto two YE5S plates at 26°C, counting the number of colonies which grew and then dividing the total by two for an average and calculating the viability by calculating this number as a percentage of 200.

Determination of septation index

Cells were fixed by mixing 850μl of culture with 150μl 16% formaldehyde, leaving at 4°C for at least 10 minutes, then washing cells and resuspending in PBSA. Cells were then stained with calcofluor, viewed under a fluorescence microscope and the presence of septa in 100 cells determined.

Drug treatment and sulphur source experiments

All drugs were added to YE5S plates. The following concentrations were used 0.5mM cadmium sulphate, 1M potassium chloride, 0.5mM tert-butylhydroperoxide, 10mM hydroxyurea, 20μg/ml thiabendazole, 0.02% methyl methanesulphonate, 50μM sodium selenate, 12.5mM caffeine or 15μg/ml cyclohexamide. For UV sensitivity cells were spotted onto YE5S plates then exposed to 250 or 500 J/m^2 UV irradiation (in a Stratagene UV Stratalinker 1800). Strains being tested were grown to exponential phase in YE5S liquid media at 26°C, then cell number counted and used to calculate serial dilutions so that 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 cells could be plated in spots on the plates.

For the cadmium physiology experiments cells were grown at 26°C in YE5S liquid media until exponential phase. 0.5mM cadmium sulphate was then added

to cultures and samples taken every two hours. For each sample cell number, viability and septation index were measured as described above and cell morphology recorded using a light microscope.
For the sulphur source experiments sulphur-free EMM was used, see section 8.1, with 5mM sodium sulphate or L-methionine added and strains were patched.

Measuring cell size

Cells stained with calcofluor were observed under a fluorescence microscope and pictures taken. Pictures were printed onto paper. Cell lengths were measured using a scale which had been previously determined by measuring 200 wildtype cells. Care was taken to ensure pictures were taken and printed using a constant scale, so that all measurements were comparable.

8.3 Molecular biological techniques

Molecular biological techniques were carried out as described (Maniatis, Fritsch et al. 1982) including preparation of competant bacteria, growth and transformation of bacteria, restriction enzyme digests and minipreps of plasmid DNA from bacteria.

Nucleic Acid preparation and manipulation

Standard molecular biological techniques were used as described (Sambrook, Fritsch et al. 1989). Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly MA and Takara Shuzo Co. Kyoto Japan). Restriction digests were performed at 37°C for 2-4 hours. DNA fragments were examined on a 1% (w/v) agarose gel in 1x TBE or TAE buffer. Ethidium

bromide was added to gel for visualisation of bands (0.5μg/ml). Electrophoresis was performed at 100V in 1x TBE or TAE buffer.

Isolation of genomic DNA from fission yeast

Yeast colonies were grown in YE5S. Genomic DNA was extracted following procedure described in Methods in Yeast Genetics, Cold Spring Harbor Press 1997.

Isolation of plasmids from fission yeast

Transformant fission yeast colonies were grown in EMM lacking the appropriate supplement for plasmid selection. Plasmids were isolated as described previously (MacNeill and Fantes, 1993), by digesting the cell wall in SZB buffer, lysing the cells in 0.1% SDS/TE pH8.0, and then precipitating cell debris by adding 5M potassium acetate. Resulting DNA was purified using Wizard DNA clean-up system, following recommended protocol (Promega), followed by ethanol precipitation as described below. Plasmid concentrations were determined by running 1μl of product on a 1% agarose/TBE gel.

Transformation of *E.coli*

Competant *E.coli* that had been stored at –70°C were thawed on ice for 20 minutes. They were then incubated with 1μl (approximately 10ng) of plasmid DNA. After heat-shocking for 90 seconds at 42°C 1ml of LB was added to each transformation and cells were incubated at 37°C for 20 minutes. These cultures were then used to directly inoculate LB containing ampicillin (100μg/ml) for plasmid amplification or plated directly onto LB agar plates containing ampicillin (100μg/ml) for subcloning.

Isolation of plasmid DNA from E.coli

400ml of transformed E.coli were grown overnight in LB containing ampicillin (100μg/ml). Cells were harvested by centrifugation (5000rpm, 5 minutes) and

the pellet was resuspended in 10ml plasmid preparation solution (P1) containing 100μg/ml RNAse. 10ml P2 solution was added and the cells left to lyse on ice for 10 minutes. 10ml P3 solution was then added to precipitate DNA. Cell Debris was removed by filtering DNA through a syringe (QIAGEN). Plasmid DNA was collected in a column (QIAGEN tip 500) which had been previously equilibrated with Equilibration buffer. The tip was then washed twice in 60ml Wash buffer, and eluted with 15ml Elution buffer. Finally isopropanol precipitation was used to purify DNA (add 0.6x volume isopropanol and wash precipitate in 70% ethanol) and DNA air-dried and resuspended in 1x T.E. buffer

Polymerase Chain Reactions

PCRs were performed with Vent polymerase when the product was to be used for transformations and with Taq polymerase for colony PCR. Vent reactions were performed in 100μl volumes with 1mM $MgSO_4$, 1μM of each primer, 10ng of template DNA, 200mM dNTPs and 2.5 units of enzyme. Products were purified using ethanol precipitation (Add 0.3M NaAc, 2.5 x volume 100% ethanol, leave at –20°C for at least 2 hours, then centrifuge, wash with 70% ethanol and leave to air-dry) and run on a 1% agarose-TBE gel to confirm size of product. For colony PCR reactions were set up as above but in a 50μl volume. A toothpick-full of yeast colony was added just before starting the reactions, and 10μl product was run directly on a 1% agarose-TBE gel with no prior purification. For subcloning a gene the Expand High Fidelity PCR System was used for gene amplification, following recommended protocol and using approximately 1μg genomic DNA as a template. All PCRs were performed in a Peilter Thermal Cycler-200

For the sequencing of mutant alleles, genomic DNA was extracted as described below, mutant locus was amplified using PCR and cycle sequencing was performed in a Peilter Thermal Cycler-200 using ABI prism

dye terminator cycle sequencing ready reaction kit and followed by automated read out using a Perkin Elmer sequencer, ABI prism 377.

Gene disruption and tagging

All genes were disrupted using PCR generated fragments (Bähler, J. et al., 1998). The 1.8 kb *ura4*$^+$ or 1.6 kb *kan*^{r} gene was amplified with flanking sequences corresponding to the 5' and 3' ends of the relevant genes. Ura$^+$ or G418^r colonies were selected on minimal plates lacking uracil or rich plates containing G418 respectively. Diploid strains, if used, were sporulated on plates lacking nitrogen and tetrad dissection of 20 asci for each diploid strain performed. Correct disruption of genes of interest was verified by colony PCR. C-terminal tagging of genes with 3HA, 13myc, GST or GFP epitopes was also carried out using PCR generated fragments (Bähler, J. et al., 1998). All tagging was confirmed by colony PCR and immunoblotting with specific antibodies.
Overexpression of Zip1 by chromosomal integration of the thiamine repressible strong *nmt* promoter in front of the *zip1*$^+$ gene was performed using the same method (Bahler, J. et al., 1998) and again transformants selected using G418 plates and correct integration confirmed using colony PCR. To observe the effects of Zip1 overexpression strains were patched onto EMM plates in the presence or absence of thiamine (150µl of 2mM thiamine was spread directly onto plates just prior to patching).

Isolation of *pof1* temperature-sensitive mutants

Error-prone PCR was used to create fragments containing randomized mutations (Cadwell, R.C. and Joyce, G.F., 1992; Cadwell, R.C. and Joyce, G.F., 1994; Lehmann, A. et al., 2004) using a *pof1*$^+$*-GFP-kan*^{r} cassette as template. Taq polymerase and buffer were used, but reactions were made mutagenic by supplying 10x dGTP (2mM per reaction) compared to dATP, dCTP and dTTP, and by supplying 5mM $MgSO_4$ per reaction. This PCR product was used to transform wild type cells. The ts *pof1-6* and *pof1-12* strains were

transformed with plasmids containing the *pof1*$^+$ gene, by which the temperature sensitivity was confirmed to be *pof1*-dependent.

Subcloning genes

For the production of *pof1*$^+$ and *zip1*$^+$ multicopy plasmids, genes were amplified using Expand High Fidelity PCR System as described above. Product was purified by ethanol precipitation, digested using appropriate enzymes and supplied buffers as described above and then DNA purified on a 1% agarose-TAE gel, by cutting out band from gel (under UV illumination) and using Gene-Clean kit (Qbiogene, Inc) following recommended protocol. pREP1 and pREP2 vectors (Basi, G. et al., 1993; Maundrell, K., 1993) were digested and purified using the same method and vector and fragments were ligated using Ligation Kit Version 2.1 (Takara Shuzo Co. Kyoto Japan) at 16°C overnight. Ligation reaction was then transformed into *E.coli* using the protocol described above, transformants selected, cultured to amplify plasmid, plasmid extracted and then tested using restriction digests to ensure it contained the correct insert.

Suppressor cloning

100ml of strain carrying *sup9* and *leu1* mutation was grown in rich media overnight until concentration was approximately $5x10^6$ cells/ml. This was transformed with 10µl of pAL library (Tanaka, Yonekawa et al. 2000), a genomic library carrying the *LEU2* marker gene. Cells were plated onto EMM plates lacking a leucine source and left at 26°C. After transformants grew cells were replica-plated onto YE5S plates containing Phloxine B and 1mM cadmium sulphate and again onto EMM plates lacking leucine to confirm they were leu$^+$. Colonies which could grow on both plates were selected, plasmid-dependency tests carried out, then plasmids extracted and sequenced as described above.

8.4 Protein Biochemistry

Western blot method

Soluble cell extract was prepared using glass beads disruption in RIPA lysis buffer or HB buffer, following the method previously described (Moreno, S. et al., 1991a). Following lysis with glass beads the extract was clarified and the protein concentration measured using the Bradford Assay (BioRad, Hercules, CA). 30μg of protein extracts were boiled for 4 minutes in SDS-PAGE loading buffer. 10% SDS-polyacrylamide gels (BioRad) or 4-20% PAGEr Duramide gradient gels (Cambrex) were used.

Proteins were then transferred onto Immobilon-P (Millipore) PVDF transfer membrane and membranes blocked in 3% milk PBSA solution containing 0.01% Tween, Primary antibodies were added in the same milk/PBSA/Tween concentration. Horseradish peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG (BioRad, Hercules, CA) and a chemiluminescence system (Enhanced Chemiluminescence Amersham plc, Little Chalfont, Bucks, UK) were used to detect bound antibodies.

Primary antibodies used for Western blots:

Mouse monoclonal:

Anti-HA antibody (16B12, Berkeley Antibody)

Anti-GFP antibody (7.1 and 13.1, Roche Applied Science)

Anti-GST antibody (G-7781, Sigma Co.)

Anti-Cdc2 antibody (Y100, Dr H. Yamano)

Rabbit polyclonal:

Anti-HA antibody (HA.11, Berkeley Antibody)

Anti-GFP antibody (Molecular Probes)

Anti-Cdc2 (phospho) (New England Biolabs)

Anti-Skp1 antibody (Dr A. Lehmann)

Immunoprecipitations

Extracts were made in RIPA buffer as described above. 2mg of these cell extracts were incubated with 3μg of polyclonal anti-HA antibody or anti-GFP antibody 1 hour at 4°C (on a rotating wheel). A 30μl slurry of Protein A-Sepharose beads (Affi-prep ® BioRad) that had been pre-equilibrated in RIPA buffer was added to the precipitation for a further 1 hours incubation at 4°C. Beads were then washed 7 times in 1ml RIPA buffer, and precipitate eluted from beads by boiling in 15μl SDS PAGE loading buffer for 4 minutes. 10μl samples were then loaded onto SDS gels and blotted with monoclonal antibodies as described above.

Quantification of immunochemical assays

The image processing program ImageJ was used to quantify the intensity of bands resulting from western blots after immunoprecipitation. Densities of bands from a scanned western blot were measured. All values were normalised against background signal.

λ–phosphatase treatment

The dephosphorylation of Zip1 in binding assays was carried out by incubating the Zip1-HA bound to anti-HA antibody and Protein A-Sepharose beads in 1x λ–phosphatase buffer containing 80 units of λprotein phosphatase. Zip1-HA was immunoprecipitated from extracts as described above, but at the washing stage beads were washed three times in RIPA buffer, incubated with λ-phosphatase for 30 minutes at 37°C, then washed four more times in RIPA buffer. 10μl extract was loaded as described above.

Detection of Zip1 ubiquitylation

An *mts3-1 zip1*$^{+}$*-HA* strain was transformed with plasmids containing a gene encoding 6His-ubiquitin under the *nmt1* promoter (pREP1-6His-Ubi). Cells

were cultured at 26 °C in the absence of thiamine and shifted to 36°C for 1 h. Cell extracts were prepared and purified by Ni^{2+}-NTA beads as described (Shiozaki, K. and Russell, P., 1997). Precipitated proteins were immunoblotted with anti-HA antibody to examine whether Zip1-HA was ubiquitylated.

8.5 Microscopic analysis

Analysis of cell morphology was carried out using cells fixed with formaldehyde. 850μl of culture was mixed with150μl 16% formaldehyde for 10 minutes at 4°C. Cells were washed with PBSA, resuspended at a covenient concentration and viewed under a fluorescence microscope with the addition of calcofluor to stain septa, or brightfield images taken using a light microscope or phase contrast microscopy.
For GFP images, Pof1-GFP strain was grown in YE5S at 22°C. Cells were washed twice in H_2O, then resuspended in Hoechst 33342 (Molecular Probes) dye solution (2μg/ml) and Hoechst staining and GFP signal viewed immediately under a fluorescence microscope.

8.6 DNA microarray experiments

As described in Chapter 6, I did not personally carry out DNA microarray experiments. Dr D. Chen in the laboratory of Dr J. Bahler at the Sanger Centre, Cambridge, UK. carried out all practical aspects of the experiment. The method used was based on that described (Chen, Toone et al. 2003) and was as follows: a wild type strain and a strain containing the zip1 missense allele were grown in YE medium at 30°C until reaching OD600= 0.2 (approximately 4 x 106 cells/ml). Cadmium sulphate was added to a final

concentration of 0.5mM, and cells were harvested at 0, 15 and 60 minute timepoints.

Total RNA was isolated using a standard hot-phenol method (for details, see the website at http://www.sanger.ac.uk/PostGenomics/S_pombe/). 20mg of total RNA was labelled by directly incorporating Cy3- and Cy5-dCTP through reverse transcription and the resulting cDNA was hybridized onto DNA microarrays containing probes for 99.3% of all known and predicted fission yeast genes printed in duplicate on to glass slides. Microarrays were scanned using a Gene Pix 4000B laser scanner (Axon Instruments, Foster City, CA) and analyzed with Gene Pix Pro software. Unreliable signals were filtered out, and data were normalized using a customized Perl script (Lyne, Burns et al. 2003). After data acquisition and within-array normalization, the ratios of each gene were divided by the corresponding ratios of untreated wild-type cells. Thus, the reported ratios represent the expression levels at each time point relative to the expression levels of the untreated wild-type cells.

8.7 Strains used in this thesis

Strain name	Genotype	Derivation
Wildtype strains		
22	*h⁻ leu1*	Lab stock
513	*h⁻ leu1 ura4*	Lab stock
TP108-3D	*h⁻ leu1 ura4 his2*	Lab Stock
CHP428	h⁺ *leu1 ura4 his7 ade6-m210*	Dr C. Hoffman
CHP429	h⁺ *leu1 ura4 his7 ade6-m216*	Dr C. Hoffman
Chapter2		
SKP407	*h⁻ leu1 ura4 pof1⁺-GST-kan^r*	Dr S.Katayama

SKP414-17	*h⁻ leu1 ura4 pcu1⁺-13MYC-kan^r*	Lab stock
Pof1Δ diploid	h⁺/h⁻ *leu1/leu1 ura4/ura4*	
	his7/his7 ade6-m210/ade6-m216	
	pof1⁺/pof1::ura4	This study
CLP30	*h⁻ leu1 pof1⁺-GFP-kan^r*	This study
Chapter 3		
CLP30-6	*h⁻ leu1 pof1-6-GFP-kan^r*	This study
CLP30-12	*h⁻ leu1 pof1-12-GFP-kan^r*	This study
CLP32-6	*h⁻ pof1-6-GFP-kan^r*	This study
CLP32-12	*h⁻ pof1-12-GFP-kan^r*	This study
CLP32-6	*h⁺ade6-M216 leu1 his2*	
	pof1-6-GFP-kan^r	This study
CLP33	*h⁻ ura4 leu1 pof1-6-GFP-kan^r*	This study
wee1	*h⁺ wee1-50*	Dr P.Nurse
wee1,mik1	*h⁻ leu1 ade6-M210,*	
	mik1::LEU2 wee1-50	Dr P.Nurse
cdc25	*h⁻ cdc25-22 leu1 ade6-M210*	Dr P.Nurse
Chapter 4		
CLP41-9	*h⁻ pof1-6-GFP-kan^R sup9*	This study
CLP42	*h⁺leu1 his2 pof1-6-GFP-kan^R*	
	sup9	This study
CLP52-2B	*h⁻ leu1sup9*	This study
CLP52-2D	*h⁺ his2 leu1 sup9*	This study
TP108-3C	*h⁻ leu1 ura4 pap1::ura4⁺*	Lab stock
CLP64-1	*h⁺ leu1 ura4 his2 zip1::ura4⁺*	This study
CLP66-1	*h⁺ leu1 his2 ura4*	
	pof1-6-GFP-kan^r zip1:: ura4⁺	This study

CLP55-2	*h⁻ kan^R-P3nmt1-zip1$^+$*	This study
Chapter 5		
mts3	*h⁻ leu1 ura4 mts3-1*	Dr C. Gordon
CLP057-2A	*h⁻ leu1 mts3-1 zip1-3HA-kan^r*	
	pof1-6-GFP-kan^r	This study
CLP053-5	*h⁻ leu1 ura4 pof1-6-GFP-kan^r*	
	zip1$^+$-3HA- kan^r	This study
CLP060	*h⁻ leu1 ura4 pof1$^+$-GFP-kan^r*	
	zip1$^+$-3HA- kan^r	This study
CLP054-5	*h$^+$ leu1 ura4 his2*	
	zip1$^+$-3HA- kan^r	This study
CLP056-5C	*h⁻ leu1 mts3-1*	
	zip1$^+$-3HA-kan^r	This study
CLP063	h- *leu1 ura4 mts3-1*	
	pof1$^+$-GFP-kan	
	zip1$^+$-3HA- kan^r ^{r}	This study
Chapter 6		
met5	*h$^+$ met5-1*	Dr P. Nurse
crm1	*h⁻ leu1 crm1-809*	Prof. M. Yanagida
sty1	*h^{90} leu1 ura4 ade6-M216*	
	sty1::ura4	Dr J. Millar

Appendix A

Primer sequences and locations

Distances shown on diagrams are taken from cosmid sequence data (Sanger Centre, UK)

Disruption and tagging of *pof1*$^+$ described in Chapter 2

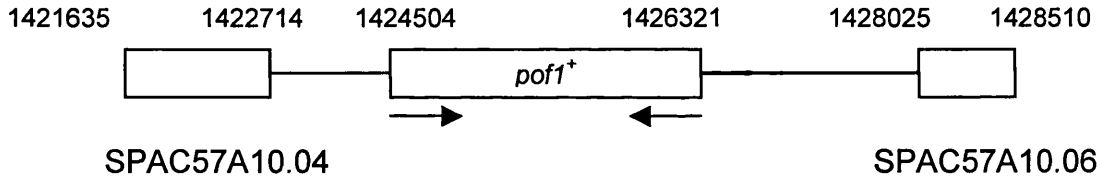
Disruption

Forward

5'ATGACTACAGGTTATGAATCTGTTCCCACTTCCGAACCCTCTGATAATC
TTGCACCACGAGCAGAGCTATGGCAACGCCAGGGTTTTCCCAGTCACGA
C

Reverse

5'CGATTGAATCGACACATCGTCATTATTGGCATTAGAGGGTGGGATATTT
AAATTTCTGGTTGAGATATTGGATAACGAGCGGATAACAATTTCACACAG
GA



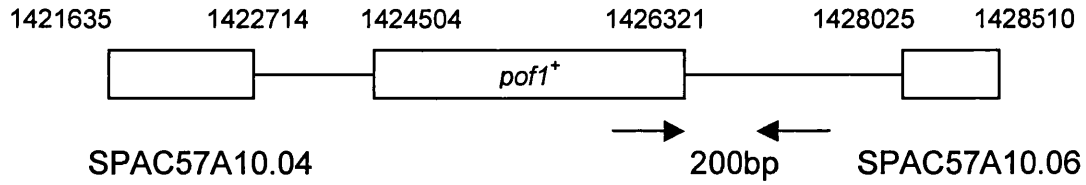
Tagging

Forward

5'-CCTCGTTATCCAATATCTCAACCAGAAATTTAAATATCCCACCCTCTAA
TGCCAATAATGACGATGTGTCGATTCAATCGCGGATCCCCGGGTTAATT
AA

Reverse

5'AGAAGTAGTTTTTGATTATAGACAAAGTGTTTGTTACCAAAAAAGAGCC
AAATTTAGATATGGCATTTTGGCAACCCGAGGAATTCGAGCTCGTTTAAA
C



Disruption and tagging of *zip1*$^+$ described in Chapter 4.

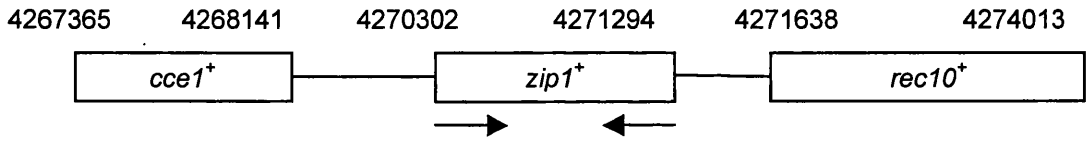
Disruption

Forward

5'ATGACTACAGGTTATGAATCTGTTCCCACTTCCGAACCCTCTGATAATC
TTGCACCACGAGCAGAGCTATGGCAACGCCAAGGGTTTTCCCAGTCACG
AC

Reverse

5'CGATTGAATCGACACATCGTCATTATTGGCATTAGAGGGTGGGATATTT
AAATTTCTGGTTGAGATATTGGATAACGAGGGATAACAATTTCACACAGG
A



Tagging-C Terminal

Forward

5'-CCATTTTAGAAACTCGTGTTCGTGAACTGGAGATGGAGAATAACTGGT
TGAAGGGACTTATACGTCCTACTTCTAACTTTCGGATCCCCGGGTTAATT
AA

Reverse

5'-GAACGTAGCAGTTGGATGCATACGCAGAAAAAAAGAAAATTAAAAGAT
TTGCTCCAAAAGAAAACATAAGTTTACCTGTAGAATTCGAGCTCGTTTAA
AC

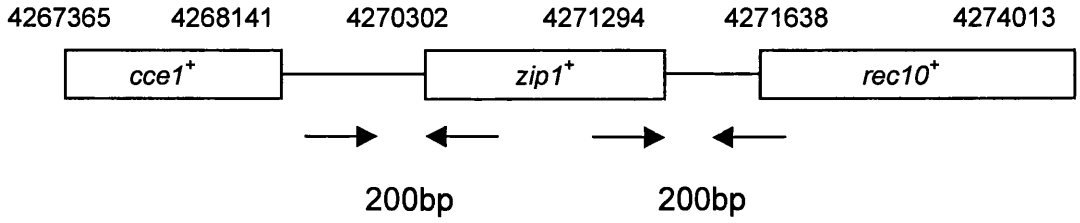
N-terminal

Forward

5'-GGTTGAGTGTGTTTTTTTTACCTTTGTGAGATATAATTTGATTAGTAA
CGATAATTTTGCCGTTAAGTCTTAGGTAAGCTGAATTCGAGCTCGTTTAA
AC

Reverse

5'-TTGAAAAATCGTCAGAGACAGGAACATCATCGAACTTAAGATTCAAG
TGATTGATAGCGCTATTAGGAGTAAAATCCATCATGATTTAACAAAGCG
ACTATA



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